Plasmids as Mediators of Gene Transfer in the Genetic Manipulation of Gram-Positive Bacteria

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ABSTRACT

Lack of suitable gene transfer techniques hampers genetic improvement and analysis of several industrially and clinically important gram-positive bacteria. Techniques already developed are often difficult to reproduce and limited in application. This study examines the feasibility of expanding the techniques available through the use of conjugation as a broad host range gene transfer mechanism. Such systems have been developed for gram-negative bacteria.

Theoretical and practical aspects, important for development of such a system for gram-positive bacteria were examined. Transformation of various gram-positive bacteria with the broad host range plasmid pAMβ1, and other vector plasmids of gram-positive origin was attempted. Conjugation of pAMβ1 from *S. lactis* to a range of gram-positive bacteria was carried out. The relationship between pAMβ1 and other plasmids was examined on the basis of DNA homology. The stability of pAMβ1 in various recipient strains was also examined. Finally, conjugation between gram negative and positive bacteria is reported for a shuttle plasmid harbouring the *mob* site of RP4. The presence of the *mob* site allows mobilization of this plasmid to streptococcal species in matings with an *E.coli* strain which has the RP4 transfer functions chromosomally integrated.

ACKNOWLEDGEMENTS

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GENERAL INTRODUCTION

Gene transfer in bacteria is a mechanism vital for studying plasmid biology and genetics. An understanding of plasmid mediated gene transfer is also a pre-requisate for the use of recombinant DNA technology. A lack of suitable gene-transfer techniques has prevented genetic analysis and genetic improvement of several important bacterial species. Genetic transfer can occur by transduction, transfection, transformation, protoplast fusion or conjugation. Genetic information can also be transferred from cell to cell by transposition events in gram-positive bacteria.

In gram negative bacteria the combination of these techniques offers versitility in manipulation of DNA, when using *E. coli* as a host. The use of broad host range conjugation systems has meant that the convenience of working in *E. coli* and the range of manipulations possible there have been coupled with work in other gram-negative species. This allowed gentic analysis which would have otherwise been very difficult. Transformation and transfection protocols have limited success in gram-negative bacteria outside *E. coli*. Transduction is limited by the knowledge of bacteriophage genetics available for particular species and even if this technique is possible, restrictions in host range and the limited amount of DNA which can be packaged limit applications.

In gram-positive bacteria, genetic systems have not been studied as well as in the gram-negatives, mainly due to the lack of suitable gene transfer techniques. There are exceptions, for example, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus lactis* and *Streptomyces* species in which much genetic work has

been completed. Nevertheless, outside these species, and usually outside particular laboratory strains of these species, the work is hampered by gene transfer limitations. The problem is all the more pressing considering that most strains of industrial importance involved in the production of enzymes and other important biologicals are gram-positive. Outside conventional mutagenesis studies most of these strains are exempted from the limitless potential for improvement which can be provided by *in vitro* and *in vivo* recombinant DNA technology. Genetic analysis of these strains is limited to cloning in other hosts, usually *E. coli*, and examinaiton of phenotypic expression in the parent species is often impossible.

In this study gene transfer techniques available for work in some important gram-positive bacteria, especially species of *Bacillus*, *Lactobacillus*, *Streptococcus* and *Clostridium*, are examined. An analysis of the potential of some novel approaches is also carried out.

INTRODUCTION:

Conjugation is the process whereby plasmid DNA is transferred from one bacterial cell to another by a mechanism which requires cell-to-cell contact. Requirement for the physical presence of the donor organism and insensitivity to DNase, allows conjugation to be readily distinguished from transduction and transformation. Conjugation allows plasmids to replicate more frequently than chromosomal genes and to transfer to alternative bacterial hosts. Small non-conjugative plasmids frequently possess an origin of conjugal transfer (ori T) and 'mobilization' genes which allow them to utilise the conjugation system of a co-existing sef-transmissible plasmid.

Originally it appeared that the transmission of genetic material by conjugative processes was restricted to gram-negative bacteria and occurred rarely, if at all, in gram-positive bacteria (Achtman & Skurray, '77). In 1964, it was suggested that conjugation might occur in gram-positives, in order to explain intraspecific transfer of chloramphenicol resistance in *Streptococcus faecalis* (Raycroft & Zimmerman, '64). In 1973, relatively high frequency transfer of a hemolysin-bacteriocin determinant was observed in *Streptococcus faecalis* (Tromura *et al*, '73). Direct evidence for a plasmid bearing this property was not provided. However, it is likely that this was the case. Direct involvement of plasmid DNA was shown when evidence for conjugal transfer of multiple drug resistance from *Streptococcus faecalis* was presented (Jacob & Hobbs, '74). Two conjugative plasmids, pJH1 (multiple resistance) and pJH2 (hemolysin - bacteriocin) were identified in this strain (Jacob *et al*, '75).

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Conjugal transfer of plasmid-encoded lactose genes between marked strains of *Streptococcus lactis* 712 (Gasson & Davies, '79, '80) and from *Streptococcus lactis* subsp. diacetylactis 18-16 into a plasmid-free derivative of *Streptococcus lactis* C2 (Kempler & McKay, '79) were also reported. Subsequent lactose plasmid transfers are reviewed by Gasson,('83 a).

Among the group N Steptococci the transfer of these and other metabolic plasmids is initially detected as low frequency events and only if donor and recipient cells have been forced together on an agar surface or membrane filter. However, some *S. lactis* ML3 conjugants that have received a lactose plasmid (33 mDal) subsequently exhibit an unusual cell aggregation phenotype and can donate the lactose plasmid at high frequency in broth culture (Walsh & McKay, '81; Snook & McKay, '81, Snook *et al*, '81). The lactose plasmid of these high frequency donors is larger (60 mDal) than the original plasmid found in the primary donor.

This larger plasmid has been shown to be a cointegrate of the lactose plasmid pSK08 and an indigenous 27 mDal plasmid of *S. lactis* ML3, pRSO1. The cointegration was *rec*-independent, occurred in random regions on pRSO1 and altered the expression of the high frequency transfer (Tra) and clumping (Clu) genes associated with pRSO1. Insertion sequences present on pSKO8 have been suggested as causing pSKO8 to integrate into pRSO1 (Anderson & McKay, 1984).

Conjugal transfer of other metabolic properties of Group N streptococci have been well documented. These include sucrose utilizing ability, nisin resistance and nisin production, plasmid encoded restriction and modification system, bacteriocin production, bacteriophage resistance and proteinase production (Gasson

& Davies, 1984 Review). All the above mentioned conjugal transfers can only take place as solid surface matings with the exception of lactose plasmid transfer from lactose transconjugants exhibiting the cell aggregation phenotype. Interspecies transfer has been confirmed to the group N streptococci.

From an evolutionary standpoint it is of interest that cell aggregation is also an established feature of a sophisticated inducible conjugation system found to operate in some *S. faecalis* medical isolates (Clewell 1981a; Review). In this, the most thoroughly studied system to date, certain relatively large plasmids (35 to 46 mDal), so far confined to strains of *S. faecalis*, transfer at very high frequencies during mixed incubation in broth culture. The system is characterized by a novel signaling mechanism whereby recipient strains excrete small peptides, or sex pheromones, which induce donors to mate.

However, the most common transmissible plasmids found in streptococci, and the only ones capable of mediating interspecies transfer, are those which require forced cell to cell contact between donor and recipient before transfer occurs. Such contact is generally facilitated by collecting a mixture of donor and recipient cells on a membrane filter, which is then incubated on a solid surface. Most, but not all, of these transmissible plasmids mediate resistance to one or more antibiotics. The best studied, the promiscuous MLS plasmids which range in size from 15 to 20 megadaltons, have been transferred to virtually every species of the streptococci (Le Blanc *et al*, 1982) and some have been transferred to other genera of gram-positive bacteria.

This type of conjugation system has been introduced to the lactic streptococci from *S. faecalis*. Plasmid pAMβ1, which encodes MLS resistance, initially

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characterized in a hospital isolate *S. faecalis* DS-5 (Clewell *et al*, 1974), is self transmissible to a wide variety of gram-positive species and genera. The plasmid was first transferred to *S. lactis* 712 and from there into and between other strains of *S. lactis, S. cremoris, S. lactis* subsp. *diacetylactis* and *S. thermophilus* (Gasson & Davies, 1980a). In addition the plasmid has been introduced into *Lactobacillus salivarius, L. acidophiles, L. casei, L. reuteri, L. plantarum* (Gibson *et al* 1979; Vescova *et al*, 1983; West and Warner, 1985) *Bacillus subtilis* (Clewell 1981b) *B. thuringensis* (Govnzales & Carlton, 1982) *B. sphaericus* (Orzech & Burke, 1984), *Staphylococcus aureus* (Engel *et al* 1980) and *Clostridium acetobutylicum* (Orltram & Young, 1985). The wide host range in which the plasmid's replication and transfer phenotypes are expressed make pAMβ1 a potentially useful plasmid both for *in vivo* strain development and as a basis for vector construction. The frequency of pAMβ1 transfer is extremely low in many *S. thermophilus* strains (Gasson & Davies 1980b) or transfer may not be detected at all, as in *L. bulgaricus* and *Pediococcus* (Gasson and Davies, 1984). Transfer frequency to *S. thermophilus* has been increased from 4.2 x 10^{-7} to 2.9 x 10^{-2} progeny per recipient by isolating mutants with increased recipient ability (Gasson & Davies, 1984). Physical and genetic analysis of pAMβ1 was carried out by Le Blanc and Lee (1984). A restriction map was plotted and the MLS determinant, the plasmid replication determinant and a region necessary for plasmid transfer were localized (see Appendix).

Another MLS resistant plasmid pIP501, extends this host range, and as well as transferring to most *Streptococcus* species (Bougueleret *et al* 1981) it also transfers to and between *Pediococcus* (Gonzales & Kunka, 1983). pIP501 also encodes chlorampenicol resistance. It has been suggested that P1P501 is a member of a family of steptococcal conjugative plasmids which share related erythromycin resistance determinants, are in the general size range of 23 - 37

kilobase (kb) and have a similar broad host range (Hershfield, 1979). Evans & Macrina (1983) have physically and genetically characterised pIP501 and constructed novel plasmids from it. Most important is pVA797 which retains transfer ability, has the Cm^R gene and the broad host range of pIP501.

Other broad host range self-transmissible plasmids of a similar class have also been reported but haven't been so well examined or as well characterised. These include pDC10535, pSM15346 (Malke, 1979) and pJH4 (Reysset & Sebald, 1985).

Mobilization in Gram-Negative Bacteria:

Conjugation in gram-negative bacteria has been reviewed extensively (e.g. Hardy, 1981). Drug resistance plasmids of the Inc P and Inc Q (e.g. RP4 & RSF1010) groups are known to be transferrable to almost all gram-negative species (Datta & Hedges, 1972). The mob regions of these plasmids have been cloned in *E. coli* vectors conferring mobilization properties on them. These constructs are not self-transmissible but contain the *ori* T of the self-transmissible plasmid and can be mobilized *in trans* if the transfer functions of the parent plasmid are supplied, either as a co-existing plasmid or immobilized in the host chromosome. It is then possible to mobilize such vectors to a wide range of gram-negative recipients and this has allowed recombinant DNA techniques to be used in bacteria which could not be transformed. This approach, useful for plasmid and transposon delivery, has been invaluable in gram-negative genetics. Recently it has been shown that using the same gram-negative mob regions plasmids can be mobilized to plant cells (Buchanan-Wolkeston *et al*, 1987) and gram-positive bacteria (Trieu-Cuot *et al* 1987a). These latter findings have significant implications for genetic exchange in nature and suggest the gene-pool of

gram-negatives is available to evolutionary distinct organisms. It has previously been shown that genes of gram-positive origin have become established in gram-negative bacteria by recent *in vivo* acquisition (Trieu-Cuot *et al*, 1987b). However, transfer of genetic information by conjugation or transduction between gram-positive and gram-negative organisms has never been obtained under laboratory conditions.

The system reported by Trieu-Cuot *et al* (1987a) which allows transfer of DNA from gram-negative to gram-positive bacteria creates the possibility of applying the mobilization technology which has greatly facilitated research in gram-negative bacteria (e.g.*Rhizobium* & *Pseudomonas* species), to gram-positive bacteria. However, this system is in it's infancy as yet, transfer frequencies are very low and the host range remains to be defined.

Mobilization in Gram-Positive Bacteria:

Transfer from gram-negative to gram-positive bacteria is a recent development and it's usefullness in DNA manipulations remains to be established. On the other hand, gram-positive conjugation systems are being adapted and analysed with respect to their usefulness in the genetic modification and analysis of gram-positive bacterial species which do not have established gene transfer techniques.

Various approaches to the use of gram-positive conjugation as a tool in molecular biology are already being developed by several laboratories. It is not yet clear to what extent the various conjugation systems can be used to mobilize non self-transmissible plasmids and the bacterial chromosome (Gasson & Davies, 1984). Mobilization of naturally occurring non self-transmissible plasmids can

certainly take place and this has been reported using conjugative lactose plasmids (Gasson & Davies, 1984) and using pAMβ1 (Daly, 1986). Plasmids of 54, 22.5 and 20 mDal coding for proteinase production in *S. crimoris* strains 17, 047 and 077 respectively, could not be transferred to plasmid free recipients using conventional agar surface mating techniques. However, these could be mobilized using pAMβ1, to an *S. lactis* SH4045 recipient, the Em^R marker being also transferred in all instances. Transconjugants were shown to contain larger novel plasmids not present in these donors. pAMβ1 was not part of the larger plasmids in strain 17 derived transconjugants (70 & 84 mDal) but was shown to be part of a 40mDal cointegrate plasmid in 047 derived transconjugants (Daly, 1986).

The mechanisms of co-mobilization involved are not known, perhaps in the case of strain 047 the resident plasmid is mobilized as a cointegrate, but sufficient evidence has not been presented to suggest mechanisms. Nevertheless, these transfers have considerable potential for the construction of new strains and the accumulation of plasmid encoded traits from various sources to single strains. This approach is already being exploited in the construction of dairy starter strains and has the added advantage that it is a natural process and is acceptable in food grade innocula. The promiscuous nature of plasmids such as pAMβ1 makes application of this type of system on a wider scale a possibility. One severe limitation of this extremely useful gene transfer mechanism is that DNA, which has been engineered *in vitro* cannot be introduced into a cell by this means.

What may happen in some cases in the above system is that a recombination takes place, between areas of homology on the self-transmissible and the non self-transmissible plasmids, resulting in transfer of the pair as a cointegrate.

Persistance as a cointegrate or resolution of the plasmids may then occur in the recipient. This type of event can indeed occur, plasmids have been constructed to this effect, and the results are indirect evidence that similar systems may operatein nature. Several plasmid vectors have been constructed using self-transmissible parent plasmids like pAMβ1 and pIP501.

Reasons for using these plasmids as a basis include; the broad host range of the replicons and antibiotic genes, the fact that deletion derivatives have been isolated during transformation and conjugation experiments and the availability of cloned segments (isolated during their genetic analysis). Cloned regions and deletion derivatives have proved very useful in vector construction (Evanes and Macrina 1983; Macrina *et al* 1982a; Simon & Chopin, personal communication). Because there is homology between these vectors and the parent transmissible plasmid it was conceivable that, when both plasmids were transferred to a *rec*-positive strain, cointegration could occur and allow co-mobilization of the vector. Such a strategy has been applied to deliver cloning vectors to non-transformable gram-positive bacteria. It involves transformation of a readily transformable intermediate host, e.g. *B. subtilis* or *S. sanguis*, with the vector and transmissible plasmid and mobilization from there to the host of interest. Cloning vectors have been delivered in this way to *L. plantarum* (Shargo & Dobrogosz, 1988) and *C. acetobutylicum* (Oultram & Young, 1987) among others. Shrago & Dobrogosz (1988) used the *E. coli - Streptococcus* shuttle plasmids pVA838 and pSA3, both constructed from pACY184 joined to pIP501 deletion derivatives. These were transformed to *S. sanguis* strains harbouring pVA797, a derivative of pIP501, where they formed cointegrates and were transferred to *L. plantarum* as deletion derivatives. The pVA797, pSA3 cointegrate failed to resolve in *L. plantarum*.

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Oultram & Young (1987) have used a different approach to mobilize vectors to *C. acetobutylicum* using pAMB1. The region of pAMB1 encoding erythromycin resistance was cloned into pJH101 (Ferrari *et al*, 1983) and this new plasmid was designated pOD1. Plasmid POD1 can replicate in *E. coli* but not in *B. subtilis*, but has a Cm^R gene which is expressed in *B. subtilis*. If pOD1 is introduced to *B. subtilis* it can only express Cm^R if a cointegrate with pAMB1 is formed, based on the homology between the Em^R genes. It was unlikely that a recombination in the Em^R region would interfere with replication and transfer functions (map , Appendix 1) and indeed it was possible to transfer such a cointegrate to *C. acetobutylicum*. More versatile derivatives of pOD1, containing multiple cloning sites, etc., have since been constructed (J. Oultram, personal communication).

A cloning strategy can be envisaged whereby the DNA is manipulated in *E. coli*, cloned into a shuttle vector like pVA838 or to a pOD type vector, transformed to a suitable intermediate host and from there transferred as a cointegrate with a transmissible plasmid to a host of interest for expression or complementation studies. Smith & Clewell (1984) have used this strategy to return *S. faecalis* DNA cloned in *E. coli* to it's original host via transformation of *S. sanguis* followed by conjugative mobilization.

It should be noted that pVA797 and pVA838 both contain the same origin of replication, a 2.8 mDal *Streptococcus ferus* plasmid and this is also the region of homology between the two. Their resulting incompatibility selects for cointegrate formation when both plasmids are selected for (Cm^R and Em^R) in the same host and probably contributes to resolution of the cointegrate and segregation of the two plasmids when selective pressure is removed. pSA3 and pVA797 contain different origins of replication and this may explain the lack of resolution

observed by Shrago and Dbrogosz (1988). It is conceivable that pAMB1 could also be used to mobilize pVA838. The Em^R gene of pVA838 is identical to that of pAMB1 (cloned during construction). But since these plasmids should be compatible the approach of Oultram & Young (1984) would seem a more effective means of employing pAMB1 as a mobilizing plasmid. Vectors based on the pAMB1 origins of replication are, however, available and may prove useful in this regard.

Smith & Cewell (1984) observed that when pVA838 was introduced to several *S. faecalis* strains from *S. sanguis*, as a cointegrate with pVA797, after resolution and segregation in *S. faecalis* no rearrangement or deletions of pVA838 occurred. However, when chimeric forms of pVA838 (with cloned fragments) were delivered to these strains transconjugants did not resolve the cointegrate at a high rate and deletions occurred. Delivery to *S. faecalis* strain M2C was employed to avert this problem. The difference in this strain remains obscure. A similar effect has been observed in *B. subtilis*. A shuttle plasmid, pDH5060, when introduced by transformation to *B. subtilis* underwent deletions when it contained inserts (Ostroff & Pene, 1983). A *B. subtilis* mutant of unknown character was isolated which could maintain the chimera intact.

The pVA838/pVA797 system has been adapted and expanded by Romeros *et al* (1979). They introduced, intact, pVA838 to several *Streptococcus* species. They also constructed a vector containing a mobilization conferring "DNA casette", incorporating the gram-positive region of pVA838 (pVA749) cloned into a polylinker to give shuttle plasmid pTG222.

The problem of resolution of cointegrates and plasmid rearrangements, especially

when DNA has been inserted, as was reported by Smith & Clewell (1984) with pVA838, might be avoided by another mobilization scheme recently reported by Smith (1985). pAM610 was constructed from pMV163 (a *S. agalactiae* plasmid which confers Tet^R) and is mobilized between streptococcal strains by pAMB1 without forming a cointegrate. pAM610 cannot be mobilized by pAD1, a pheromone dependent *S. faecalis* conjugative plasmid, between *S. faecalis* strains. A derivative of pAM610, pAM910, which lacks approximately 1kb of pMV163 (streptococcal) DNA, cannot be mobilized by pAMB1 or pAD1 (Smith, 1985).

This suggests that pAM610 can only be mobilized by the MLS class of conjugative plasmids and that a small area of the pAM610 plasmid is necessary for it's mobilization.

Yu and Pearce (1986) showed that pAM610 could be mobilized to *C. acetobutylicum* from several *Streptococcus* donors by pAMB1 and pVA797. They also loaded pVA797 with the gram-positive transposon Tn917 and it transferred at frequencies similar to wild type pVA797. The Em^R of Tn917 was expressed there and may prove a useful tool. Transposition of Tn917 was not reported.

It can be envisaged that conjugation will prove to be a very useful technique in gram-positive bacterial genetics. In the absence of other suitable gene transfer techniques, conjugation could open up these species to genetic modification and analysis. Unlike other gene transfer methods, if a conjugation strategy is developed for a particular strain using the broad host range conjugation systems, it is likely that the same approach can be used in a range of species of different genera. This type of approach has proved very useful in gram negative

bacterial genetics. It is widely accepted that despite developments in gram-positive genetics, especially with *Bacillus* and *Streptococcus* species and *S. aureus*, one of the major obstacles to rapid progress, in these species and species of other genera, is the lack of suitable gene transfer methods in most cases. This is especially true for industrial important strains which often do not respond to protocols used with laboratory strains of even the same species.

PLASMID-LESS CONJUGATION AND TRANSPOSONS

"Plasmid-less" conjugative transfer requires the confirmation of evidence of a chromosomal location of transferred genetic material either in the donor or transconjugant cells. Such evidence has been provided in a few instances among the streptococci. The conjugative tetracycline resistance (Tc^R) transposon, Tn916, from strain *S. faecalis*, is capable of self transfer in mating experiments (Gawron-Burke *et al*, 1985). This 10 Mdal transposon is capable of transposition to different conjugative plasmids. Furthermore, transposition occurs at an unaltered frequency in a recombination deficient host and so there is considerable potential for exploiting this novel type of gene transfer system which combines transposition and transfer (Gawron-Burke *et al*, 1985).

The conjugative Tc^R transposon Tn919 is similar to Tn916 and was originally isolated in *S. sanguis* FC1. It is capable of low frequency transfer on membrane filters to several lactic acid bacteria (Hill *et al*, 1985). However, the introduction of a plasmid pMG600 (Lac$^+$ Lax$^-$, a novel Lac plasmid from *S. lactis* 712, capable of conjugative transfer at high frequencies, and which in certain hosts confers an unusual clumping phenotype) into *S. lactis* CH919 donor (containing Tn919). generating *S. lactis* CH001 (containing both Tn919 and pMG600), resulted in a significant improvement in transfer frequency of Tn919 to *S. lactis* CK50. Using this technique, Tn919 also transferred at high frequency to *S. lactis* subsp. *diacetylactis* 18-16S, but not to *S. cremoris* strains. Insertion of Tn919 in *S. lactis* subsp. *diacetylactis* 18-16S transconjugants was random, occurring at different sites on the chromosome and also in plasmid DNA. Thus, the conditions necessary for the exploitation of Tn919 in targeting

and cloning of genes from a member of the lactic streptococci, namely high frequency delivery and random insertion in host DNA, have been achieved (Hill *et al*, 1985).

Gawron-Burke & Clewell (1984) described a strategy for targeting and cloning of genes from gram-positive bacteria in *E. coli* using Tn916. This was allowed by regeneration of insertionally inactivated stretptococcal DNA (transposon mutagenesis carried out in *Streptococcus*) by precise excision of Tn916 in *E. coli*. More recently (Pozzi *et al*, 1988) a similar type of conjugative DNA element (described as a conjugative chromosomal element), S2 6001, has been used for delivery and integration of recombinant DNA into chromosomes of transformable and non-transformable streptococci.

Another gram-positive transposon Tn917 has been used extensively in *B. subtilis* for genetic manipulation. (Youngman, 1983; Review). Tn917 is non-conjugative. This transposon has also been used for insertional mutagenesis and genetic analysis in *S. faecalis* (Ike & Clewell, 1984) and may prove useful in a similar role in other gram-positive bacteria.

PROTOPLASTS, PROTOPLAST FUSION AND PLASMID CURING:

Several workers have published information on the generation and regeneration of protoplasts of gram-positive bacteria. These include lactic streptococci (Gasson, 1980; Okamoto et al, 1983) lactobacilli (Chassy, 1987), clostridia (Jones and Woods, 1986) and *B. subtilis* (Chang and Cohen, 1979). Variations occur with different strains but generally high efficiencies of generation and re-generation have been reported, except perhaps with clostridia. The development of these techniques, as has already been discussed, was significant in allowing protoplast transformation protocols to be developed for *Streptococcus lactis* and *B. subtilis*, among others. Several strains which could be protoplasted could, however, not be transformed.

Protoplast fusion is an alternative method of gene transfer. Protoplast fusion may be described as the fusion of cells in a mixed population of protoplasts derived from two parental strains. The primary product of this fusion is a protoplast containing the complete genome of both parents. A detailed study in *Streptomyces coelicolor* has shown that genetic reconstruction is random and highly efficient. Subsequentially DNA is lost from the hybrid cell but traits originating from both parents may be maintained. Protoplast fusion is induced by PEG after which regeneration is allowed to take place on hypertonic medium. Intra specific and intergeneric protoplast fusions have been reported in gram-positive bacteria and offers a means of transfer of plasmid and chromosonal DNA between these, albeit limited range, of bacteria.

Smith (1985) transferred non-conjugative plasmids by protoplast fusion among *S.*

faecalis strains and from *S. sanguis* to *S. faecalis*. Gasson (1980) demonstrated plasmid transfer between industrial *S. lactis* strains by protoplast fusion. In one experiment he noted that pAMB1 could transfer between protoplasts in the absence of PEG at high efficiency and that the mechanism in this case probably involved conjugation. This is the first report of conjugation to/or between protoplasts. Jones *et al* (1985) successfully used protoplast fusion to complement auxotrophic strains of *C. acetobutylicum*. There have recently been two reports of plasmid transfer by intergeneric protoplast fusion between two phylogenetically widely separated gram-positives, *S. lactis* and *B. subtilis*.

Baigori *et al* (1988) introduced the shuttle plasmid pGK12, as well as several *S. aureus* plasmids into *S. lactis* by protoplast fusion with *B. subtilis*. All plasmids were stably inherited. Van der Vosseu *et al* (1988) have also demonstrated transfer of pGK12 and the promoter screening vector pGKV21 from *B. subtilis* to *S. lactis* by protoplast fusion at a similar frequency to intraspecific transfer. This system was developed to allow pre-cloning of *S. cremoris* specific promotors in *B. subtilis* (in the absence of a satisfactory transformation protocol for *S. lactis* and subsequent selection in *S. lactis* Cocconcelli *et al* (1986) have carried out integeneric transfer of pAMB1 from *S. lactis* to *L. reuteri* at low frequency.

Protoplast fusion is of potential value as a method of gene transfer complementary to those already discussed for gram-positive bacteria. The process is simple, unsophisticated and because fusion is neither strain-specific, species specific nor perhaps genus specific, it is widely applicable.
A further use of protoplasts is based on the observation that plasmids are often lost during protoplast regeneration in *S. aureus* (Novick *et al*, 1980). This occurrance seems to be common in gram-positive bacteria and may be due to an

involvement of the cell envelope in plasmid maintenanc and replication (Novick *et al*, 1980). Protoplast induced curing of plasmids from *S. lactis* subsp. *diacetylactis* and *S. lactis* were reported by Gasson (1983a). Partially cured strains of *S. lactis* NCDD 712 (five plasmids) and a plasmid-free strain (MG1363) were isolated. Vescavo *et al* (1984) effectively cured plasmids from *Lactobacillus reuteri* by protoplast formation and regeneration. Similar approaches have also been used for plasmid curing in *Streptomyces* species (Hopwood, 1981) and *B. subtilis* (Fowler *et al*, 1981). A recent report (Abdalla-Galal, 1988) has demonstrated the curing of plasmids from *S. aureus* strains using antibiotics affecting the bacterial cell wall. Complete curing required cell divison (L-forms, protoplasts which can undergo cell growth and division) which could be the result of an inhibition of new rounds of plasmid replication following the loss of DNA - envelope interaction.

TRANSFORMATION

Genetic transformation is a process by which a cell takes up naked DNA from the surrounding medium and incorporates it to acquire an altered genotype that is inheritable. Transformation has been studied extensively in many organisms since it was first discovered in 1928 in *Streptococcus pneumoniae* by Griffith.

Bacteria that are able to take up free DNA are said to be competent. Some bacterial strains are highly competent during one or more phases of growth under normal laboratory conditions, whereas others require special treatments to be rendered competent. Still others appear completely refractory to transformation and cannot be induced, under conditions tested, to a competent state.

Factors determiniing competence are not fully understood but lack of transformability could presumably be due to a number of factors, e.g. lack of DNA binding sites, cell wall barriers, cell surface barriers and the production of intra- and extracellular nucleases.

Bacteria that are highly transformable include *Streptococcus pneumoniae*, *Haemophilus influenzae* (Alaxander and Leidey, 1951) *Bacillus subtilis* (Spizzeen 1958) and *Streptococcus sanguis* (Challis) (Le Blanc and Hassell 1976). In competent gram-positive bacteria, e.g. *S. sanguis* Challis, DNA transformation, plasmid or chromosomal, involves binding of double stranded DNA, uptake of single strands and recombination of complementary strands of DNA (Behuke, 1981). In gram-negative bacteria the process seems to differ and DNA is taken up in the form of duplex molecules.

E. coli do not become competent naturally and special treatments are required to induce competence. This is accomplished by producing spheroplasts (Henner *et al*, 1973) or treatment with Ca^{2+} ions and subjection to heat shock which render the cells permeable to DNA. The procedures for transformation in *E. coli* have been well optimized, are efficient, and easily reproduced (5 x 10^8 trans/ugDNA). *Rhodopseudomonas sphaeroides* can be rendered competent by treatment with concentrated Tris buffer (Fornari & Kapian, 1982) while *Saccharomyces cerevisiae* can be induced to take up DNA by pre-treatment with Triton X-100 and concentrated metal ions (Kimura, 1986). Bacteria which cannot achieve natural competence and those which are refractory to induction of competence by laboratory manipulations have been transformed with plasmid DNA by removing cell wall and surface barriers (i.e. generation of spheroplasts and protoplasts) and providing specific conditions. This has been an important development in gram-positive genetics and provided the opportunity for genetic manipulation of many species which could not be induced to competence, e.g. *Streptococcus lactis* (Kondo and McKay, 1982) and *Streptomyces* (Bibb *et al*, 1978). Even with bacteria which could achieve competence, protoplasting has afforded greater versitility. For example a protoplast transformation protocol has been developed for *B. subtilis* (Chang and Cohen, 1979). The requirement for recombination of homologous single strands in competent *B. subtilis* transformation poses problems in gene cloning experiments.

Multimers which can recombine on themselves to form duplex plasmid DNA transform competent cells most efficiently. In a shotgun cloning experiment frequencies are therefore much reduced. Secondly, recombination deficient mutants of *B. subtilis* could not be transformed in this way. Also, the single strand taken up is often partially degraded resulting in deletions. These

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problems are not experienced if protoplast transformation is used.

Protoplast transformation has been attempted for various bacterial species with varying degrees of success. Early investigations into the transformation of *S. lactis* suggested that whole cell procedures were unsatisfactory (Kondo & McKay, 1982, a) Similar findings for *Lactobacillus casei* were also inferred (Lee-Windener and Chassey, 1984). Since then it has been generally assumed that transformation of the lactic acid bacteria is limited to protoplast dependent procedures and numerous studies have been carried out on a variety of lactic acid bacteria (Fujita *et al*, 1983: Kondo & McKay, 1982 a) and subsequently by several laboratories. Maximum efficiency of protoplast transformation in *S. lactis* was reported by Simon *et al*, 1986, who were able to obtain 5×10^6 erythromycin-resistant transformants per ug of pIL204 DNA. These frequencies have been very difficult to reproduce especially in other laboratories (A. Chopin, personal communication). Transformation frequencies are included in only two other reports Yu *et al*, 1984, who reported 8 transformants per ug of DNA and Kondo & McKay, 1984, who recovered 4×10^4 transformants per ug of pGB301 DNA.

Although transformation of *S. lactis* with plasmid DNA has been accomplished, the poor understanding of protoplast formation and regeneration conditions as well as DNA uptake mechanisms, has in practice caused difficulties in reproducing established protocols in different laboratories (Sanders and Nicholson, 1987). Such are the problems that recently a procedure has been reported (Sanders and Nicholson, 1987) which does not require protoplasts and yields 5×10^5 transformants per ug pSA3 DNA and the authors claim the simplified procedure will be easier to reproduce. In this technique incubation in hypertonic media and PEG were used to introduce DNA into non-protoplasted cells.

In the lactobacilli there have been no reports to date of natural competence or induced competence. A procedure for generation of protoplasts (using mutanolysin) and regeneration has been developed for *L. casei* and has been applicable to several other species of *Lactobacillus* (Chassy, 1987). Therefore, the basic requirements for protoplast transformation techniques have been met.

Despite these developments gene transfer in the lactic acid bacteria remains an obstacle in the development of lactic acid bacterial genetics. Problems of reporducibility and strain dependence of transformation protocols hampers the general applicibility of transformation as an experimental tool.

L. acidophilies can be transformed to rifampicin resistance at low frequency by homologous DNA isolated from rifampicin resistant strains (Lin and Savage, 1986). Protoplasts of *L. reuteri* have been transformed to erythromycin resistance by pSH3 (Horelli *et al*, 1987). An *L. plantarum* shuttle vector constructed from an *L. plantarum* cryptic plasmid has been transformed to *L. plantarum* (Chassy, 1987). *L. casei* protoplasts have been transformed with lactose plasmids and plasmid pLZ15 encapsulated in liposomes to good frequencies (Chassy, 1987). All of the plasmid transformations described above suffer from either low frequency, inconsistent reproducibility or both. The use of liposomes may raise the frequency. It hasn't always been possible to isolate DNA from transformants. Considering the report that formation and regeneration of *Lactobacillus* protoplasts is a very effective means to cure plasmids (Vescova *et al*, 1984) it is possible that completely protoplasted cells were forced to rescue genes by an integrative event.

Attempts have been made to transform other gram-positive bacteria of industrial importance. Transformaiton of *C.acetobutylicum* by chromosomal or plasmid DNA, via the mechanism of natural competence or by employing treatments which have facilitated the uptake of DNA by whole cells in other species, have not been successful to date. Methods for the production and regeneration of protoplasts have been reported for *C. acetobutylicum, C. pasteuriarium* and *C. saccharoperbutylacetonicum* (Allcock *et al*, 1982; Minton and Morris, 1983; Yoshino *et al*, 1984). This has opened the way for experiments on protoplast fusion and protoplast transformation. Transformation of *C. acetobutylicum* both by phage and plasmid DNA have been reported. Both systems employed approaches which resulted in a decrease of extracellular DNase activity associated with the protoplasts. Many strains of *C. acetobutylicum* seem to be associated with high levels of DNase activity which is either present throughout the whole growth cycle or associated with sporulation (Jones & Woods, 1986; Reid *et al* 1983). This activity may hamper the isolation of both plasmids and chromosomal DNA and appears to interfere with transformation. Reid *et al* (1983) transfected *C. acetobutylicum* P262J, which was derived from P262 and had lower levels of extracellular DNase activity but still exhibited a high frequency of protoplast regeneration. Lin and Blaschek (1984) successfully transformed the butanol-tolerent *C. acetobutylicum* SA-1 strain with pUB110 DNA after heat treatment of protoplast at $55^{\circ}C$ for 15 min to inhibit DNase activity. Attempts to repeat this transformation were unsuccessful in other strains of *C. acetobutylicum*, indicating that the factors involved in the transformation of different strains may be varied and complex.

Electroporation uses a high-voltage electric discharge through a suspension of cells to induce trancient 'pores' in the cell membrane through which DNA enters the cells. This technique has recently found application in the transformation of many types of eucaryotic cells, including plant and yeast protoplasts, and B lymphocytes (Shillito *et al*, 1985; Karube *et al*, 1985; Potter *et al*, 1984). *Bacillus cereus, S. lactis, L. casei, L. acidophilus* and *L. plautarum* have been transformed using electroporation (Shivarova *et al*, 1983; Harlander, 1986; Chassy & Flickinger, 1987; Aukrust & Nes, 1988.

Introduction of DNA into *L. casei* occurred rapidly and simply. A Gene Pulser^{TM} supplied by Bio-Rad Laboratories was used. The procedure involved chilling the cells on ice and adding the DNA ten minutes, or less, before subjecting the cells to a single pulse electroporation. The cells were chilled for another 10 mins on ice, diluted in liquid medium, incubated to allow expression of transformed marker, and plated. Transformation occurred at high frequency and efficiency (Chassy & Flickinger, 1987).

L. plantarum ATCC 8014 was transformed with pTV1 by electroporation using a modification of a procedure used for *E. coli*. Voltage range (5,000 - 6,250 V/cm) at constant capacitance setting of 25uF was used and not found to be critical. Incorporation of 1% glycine in the growth medium was found to be critical.

A problem with electroporation as a means of transformation is the fact that conditions worked out for one strain (voltage, capacitance, time constant) will usually not work for other strains of even the same species. However, once

conditions are optimized the technique is reasonably reproducible (Ger Fitzgerald, personal communication). It appears that different chemical treatments and growth phase of the cells may also be important. Some workers have spent a lot of time varying conditions without achieving successful electroporation (Badii and Warner, 1988). Use of protoplasts may also assist electroporation (Badii and Warner, 1988; Karuibe *et al*, 1985). Perhaps the technique of electroporation will not be as easily applied to prokaryotes as was initially envisaged.

TRANSDUCTION

Transduction is the transfer of bacterial DNA from a donor cell to a recipient by bacteriophage capsid. The transducing particle retains the adsorption and infection mechanisms of the normal phage. Once the DNA has entered the recipient cell three pathways are possible: if the DNA is a replicon (e.g. a plasmid), it can be inherited intact; if it is a fragment of DNA stable inheritance requires recombination with a recipient replicon; if neither of these events occur the DNA can survive for a period without replication, but will eventually be degraded. There are two classes of transduction termed specialized and generalized transduction.

Specialized transduction occurs when the transducing particle carries the bacterial DNA as an insertion or substitution within the viral genome, due to imprecise excision of the prophage from the bacterial DNA upon induction of vegitative phage replication. Only bacterial DNA flanking the prophage can be transduced in this way. In contrast, generalized transduction appears to be the accidental, packaging of bacterial DNA inside a phage capsid - the transducing phage contains only, or almost only, bacterial DNA and any segment of the bacterial genome can be encapsulated.

Phages containing double stranded DNA have been detected in a wide variety of bacteria but those capable of transduction have been detected in a more limited number of genera. All phages do not have transducing capability. Packaging may not occur or may not be possible in cases where phage infection leads to a rapid and extensive degradation of host DNA. Adsorption must not interfere with cell viability. With some phages adsorption is sufficient to cause cell

death. The injected DNA must survive the barrier posed by nuclease degradation in the recipient. Some phages have a broad host range but more usually transduction is confirmed to the same species but can be extended to a large number of strains if effects of DNase can be overcome. The size of DNA that can be transduced is limited by the DNA capacity of the phage. Plasmids of smaller or similar size can be transduced intact while the process selects for spontaneously arising deletion derivatives of larger plasmids. Size range packaged is usually in the range 20 to 70 mD. Transductional studies are limited because of the unavailability of documented transducing phages for many species and also because of the lack of virulent or temperate phages whose transductional capabilities might be tested in the case of many species.

The first account of transduction in lactic streptococci mediated by temperate bacteriophage involved *S. lactis* C2. Spontaneous Lac⁻ *S. lactis* C2 was transduced to Lac⁺ phenotype using temperate bacteriophages which were induced from a Lac⁺ *S. lactis* C2 wild type culture by exposure to u.v. irradiation. The number of transductants generated by this method was low. However, a lysate prepared from a Lac⁺ transducant was capable of transferring this marker at a 100-fold increase in a high frequency transduction (HFT) (McKay *et al*, 1973). Lysogenic phage preparations were also capable of transducing the maltose and mannose markers to mutants of *S. cremoris* C2 but neither of these exhibited the HFT phenomenon (McKay & Baldwin, 1974) and neither was found to be unstable, indicating chromosomal linkage of these markers (McKay & Baldwin, 1974).

It has been found that in some cases the *lac* genes could integrate into the chromosome after transduction, thereby stabilising the Lac⁺ phenotype (McKay & Baldwin, 1978). Plasmids of novel sizes (20 and 23 Mdal) have also been seen

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in transductants. McKay *et al* (1980) postulated that these may have arisen due to transductional shortening of the molecule to enable it to be packaged into the phage head.

The availability of prophage-cured derivatives of *S. lactis* 712, which act as indicators for temperate phage, has facilitated research on transduction in this strain (Gasson & Warner, 1982). Results, similar to those using *S. lactis* C2, were obtained. Chromosomal maltose genes and extrachromosomal lactose and erythromycin genes were transduced, some of the lactose transducants also receiving the proteinase determinant. Analysis of the plasmid complements of *S. lactis* 712 transducants revealed novel lactose plasmids of 22 Mdal in most strains, indicating that a larger unresolved lactose plasmid had been transductionally shortened. Integration of *lac* genes into the chromosome has also been reported (Gasson & Warner, 1982). It has been shown, on the basis of restriction enzyme analysis, that the novel *lac* plasmids contain no phage DNA. This evidence tends to rule out the theory that the HFT phenomenon is due to recombinatiron between the plasmid and phage DNA. It has been suggested that the HFT phenomenon is due to phage heads which preferentially package "shortened" *lac* plasmids and that phage will package preformed shortened plasmids rather than effecting the shortening process itself (Kando & McKay, 1984). In *S. lactis* C2 and 712, few *lac* plasmids will be spontaneously deleted to 20-23 Mdal, thereby accounting for the low frequency of transducitron. However, novel *lac* plasmids in the transductants are within the correct size range to be packaged by the phage and hence give rise to the HFT phenomenon.

Transduction has also aided in evaluation of a recombination-deficient (*rec⁻*) mutant of *S. lactis* ML3 (Anderson & McKay, 1983b). Recombination is a

mechanism by which the genetic information can be introduced into host DNA by physical insertion. The process of insertion usually requires breakage of recipient DNA and the rejoining of donor DNA to recipient DNA. The *rec* genes are responsible for this type of recombination and introduction of chromosomal genes into cells is *rec*-dependent. A *rec⁻* mutant (MMS36) was isolated using a technique based on characteristic sensitivities to u.v. irradiation and mutagens. Confirmatiron of the *rec⁻* phenotype was achieved by transduction of chromosomally-mediated streptomycin resistance (Sm^R) and plasmid-coded lactose metabilism to this strain. Sm^R transductants of ML3 were readily observed but no Sm^R transductants of MMS36 were obtained. Transduction of plasmid coded lactose metabolism was successful in both strains. The inability of the phage to transduce chromosomal markers to MMS36 confirmed that MMS36 was *rec⁻*. The strain MMS36 can be used to distinguish plasmid and chromosomally-coded traits (Anderson & McKay, 1983, b).

Fermentations by lactobacilli are not frequently hampered by phage infections; there appear to be fewer *Lactobacillus* phages and more so that the dairy streptococci, many strains appear to be either phage resistant or are immune lysogens. However, phages have been reported in a number of species of lactobacilli (Yokokura *et al*, 1974; Coetzee *et al*, 1962). Two phages of *L. casei* have been extensively characterized and the genome of an *L. bulgaricus* phage has been analysed by molecular cloning in *E. coli* (Chassy, 1987). But as yet, as of this writing transduction of lactobacilli has not been reported.

Although transduction has not been reported in *C. acetobutylicum* both lytic and lysogenic phages have been isolated from a number of strains of saccharolytic solvent producing clostridia. The first isolation of a phage from an abnormal fermentation broth was first reported in 1944 and phage infections in industrial

fermentations seem to have been a common occurrance (Jones and Woods, 1986). The characteristics of a number of phages which were isolated from solvent-producing clostridia have been reviewed (Ogata & Hongo, 1979). Because of the availability of phages for examination in *C. acetobutylicum* it may be feasible to develop transduction systems or phage cloning vectors (similar to ϕ105 in *B. subtilis*) for *C. acetobutylicum*.

MATERIALS AND METHODS

M. 1. BACTERIAL STRAINS AND PLASMIDS

TABLE 1

Bacterial Strain	Genotype/Phenotype	Source
E. coli C600	*thr, leu, thi,* *hsd,* M$^+$, *psl.*	NIHE culture collection.
JA221	F^1 *rec*A1, *leu*Bb, *trp* E5, *hsd*M+, *hsd*R$^-$, *lac*Y, *xyl,* C600.	NIHE culture collection.
C600 (pGK12)	C600 transformed with pGK12.	This study.
S17-1		NIHE culture collection.
DH1 (pAM401)		C. Geoghegan.
S. lactis MG1363	Plasmid free, Sm^{600}, *S. lactis* 712.	UCC culture collection.

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TABLE 1

Bacterial Strain	Genotype/Phenotype	Source
MG3020	Mg1363 containing pAMβ1.	UCC culture collection.
MP205	MG1363 containing pGK12.	C. Geoghegan.
MG1363 (pAM401)		This study.
MG1363 (pAM401-mob)		This study.
MG3020 (pAMβ1, pAM401)		This study.
MG 3020 (pAMβ1, pAM401-mob)		This study.

TABLE 1

Bacterial Strain	Genotype/Phenotype	Source
Bacillus subtilis 168 Met A	*met*A	TCD culture collection.
SO113 TrpC2	*trp*C2	TCD culture collection.
168 Met A (pAMβ1)	pAMB1 transformants and transconjugants containing deletions in pAMB1.	This study.
168 MetA (pAM401)	pAM401 transformants.	This study.
168 Met A (pIL 253)	pIL253 transformants.	This study.

<u>TABLE 1</u>

<u>Bacterial Strain</u>	<u>Genotype/Phenotype</u>	<u>Source</u>
S. faecalis		NIHE culture collection.
B. thuringensis		NIHE culture collection.
C. acetobutylicum		UCG culture collection.
C. butyricium		UCG culture collection.

M2.   <u>MEDIA</u>

<u>GM17 Broth</u>:

(Based on M17 described by Terzaghi & Sandine, 1975).

Phytone Peptone	5g
Polypeptone	5g
Yeast Extract	2.5g
Beef Extract	5g
Glucose	5g
Ascorbic Acid	0.5g
β-Disodium Glycerophosphate	19g
IM $MgSO_4.7H_2O$	1ml
H_2O	1 litre

<u>SGM17 Broth</u>:

GM17 made 0.5M with respect to sucrose.

Solid Media were prepared by the addition of 1.5% agar.

Soft medium for overlay was prepared with 0.5% agar.

<u>Brain Heart Infusion (BHI)</u>:

As supplied by Oxoid.

Reinforced Clostridia Medium (RCM):

As supplied by Oxoid.

Luria Broth (LB):

Bacto-Tryptone	10g
Bacto-Yeast Extract	5g
NaCl	10g
H_2O	1 litre

pH adjusted to 7.5 with NaOH.

MRS Medium:

As supplied by oxoid.

Clostridium Minimal Medium (MMC):

Glucose	20g
NH_4Cl	3g
$MgSO_4.7H_2O$	0.4g
$CaCl_2:6H_2O$	2.2g
p-Aminobenzoic acid	200ug
Biotin	12ug
Potassium Phosphate (pH 7.0)	50mM
Trace Element Solution *	2ml
H_2O	1 litre

Adjusted to pH 6.0 with 2M KOH.

(Modification to trace element solution of Vishniac & Santer, 1957.)

EDTA	50g
$ZnSO_4.7H_2O$	2.2g
$CaCl_2$	5.54g
$MnCl_2.4H_2O$	5.06g
$FrSO_4.7H_2O$	4.99g
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.10g
$CaSO_4.5H_2O$	1.57g
$CoCl_2.6H_2O$	1.61g
H_2O	1 litre

Adjust pH to 6.0 with 2M KOH.

Glucose-Glutamate Minimal Medium for *B. subtilis* (MMB):

(Modification of Pigott *et al*, 1973)

$FeCl_2.6H_2O$	0.98mg
$MgCl_2.6H_2O$	8.3mg
$MnCl_2.4H_2O$	19.8mg
NH_4Cl	535.0mg
Na_2SO_4	106.0mg
KH_2PO_4	68.0mg

NH_4NO_3	96.5mg
$CaCl_2$	219.0mg
L-Glutamate	2.0g
Glucose	2.0g
L-alanine	100.0mg
H_2O	1 litre

pH adjusted to 7.1 with 2M KOH.

(L-amino acids added as required by auxotrophs.)

B. subtilis Transformation Medium (TM):

(Added to 10ml plastic tube.)

1 x spizzen salts (SS)	5ml
40% glucose	50ul
2% casamino acids	50ul
Amino acid required by auxotrophs (10mg/ml solution)	25ul
10% yeast extract	50ul
0.1M $MgCl_2$	125ul
50mM EDTA	200ul
0.05M $CaCl_2$	50ul
Total volume	5.5ml

Spizzen Salts (SS):

K_2HPO_4	14.0g
KH_2PO_4	6.0g
Ammonium sulphate	2.0g
Sodium Citrate.$2H_2O$	1.0g
$MgSO_4$	0.2g
Glucose	5.0g
H_2O	1 litre
pH	7.0

SP I Medium (for generation of competent *B. subtilis*):

1 x spizzen salts	500mls
40% glucose	5mls
Amino acid required by auxotroph (10 mg/ml)	2.25mls
Casaminoacids (2%)	5mls
Yeast Extract (10%)	5mls
Total volume	517.25mls

SP II Medium:

SP 1	500mls
0.1M $MgCl_2$	12.5mls
0.5M $CaCl_2$	5.0mls
Total volume	517.5mls

M. 3.   BUFFERS AND SOLUTIONS

TE Buffer:

Tris-HCl	10 mM
Na_2-EDTA	1 mM
pH	8.0

TES Buffer:

Tris-Hcl	10 mM
Na_2-EDTA	1 mM
NaCl	50 mM
pH	8.0

For gel electrophoresis:

Tris borate buffer   (10 x concentrate):

Tris-HCl	108 g	
Na_2-EDTA	9.3 g	
Boric Acid	55.0 g	
H_2O	1	litre
pH	8.3	

Tris acetate buffer:

Tris-HCl	40 mM
Na-acetate	10 mM
Na_2-EDTA	1 mM
pH	7.8

For southern Blotting:

Denaturing Solution:

NaCl	1.5 M
NaOH	0.5 M

Neutralization Buffer:

Tris-HCl	1.0 M
NaCl	1.5 M

SSC (20 x concentrate):

NaCl	3.0 M
Na-citrate	0.3 M

For Nick Translation:

Nick translation buffer (10 x concentrate):

Tris-HCl (pH 7.0)	0.5	M
MgCl$_2$	0.1	M
DTT	10	M

DNAase 1 was diluted in nick translation buffer with 50% glycerol and stored at -20°C.

For Hybridization:

Denhardt's Solution:

Serumalbumin	0.1	%
Polyvinylpyrolidon	0.1	%
Ficoll	0.1	%

For Autoradiography:

Developer:

Kodak LX24

Developer   100 ml

H_2O 600 ml

Fixing bath:

Kodak FX40

Fixer   200 ml

H_2O 800 ml

For DNA Digestion and Ligation:

Restriction buffers supplied with enzymes were used in general except with double digests when enzymes being used required buffers of different salt strength. In such cases, following restriction with the low salt buffer, salt concentration was adjusted as required and the high salt buffer requiring enzyme added. One hour was allowed for completion of each restriction.

Ligation buffer:

Tris-HCl pH 7.5	0.5	M
$MgCl_2$	0.1	M
DTT	0.1	M
Spermidine	1	mM
ATP	10	mM
BSA	1	mg/ml

(Stored at -20°C.)

Calf Intestinal Phosphatase buffer (CIP) (10 x concentrate):

Tris-HCl pH 9.0	0.5	M
$MgCl_2$	10	mM
$ZnCl_3$	1	mM
Spermidine	10	mM

STE buffer (10X):

Tris-HCl pH 8.0	0.1	M
NaCl	1	M
Na_2-EDTA	10	M

For DNA Isolation and Preparation:

STET Buffer:

Sucrose	8 %
Triton X100	5 %
EDTA	50 mM
Tris-HCl, pH 8.0	50 mM

Kirby Mix:

Phenol	50 ml
Chloroform	48 ml
Iso-amylalcohol	2 ml

For *S. lactis* Protoplast Generation:

SGM17-protoplast buffer:

GM17 with 0.5 M sucrose and add 1/10 volume of 10X protoplast buffer salts.

Protoplast Buffer Salts:

Ammonium Acetate	0.4 M
Magnesium Acetate	0.01 M
Sucrose	0.5 M

For Transformation:

SMM-Ca^{+2}:

Sucrose	0.5 M
CaCl$_2$	0.05 M
Malate	0.02 M
pH	6.5

M. 4.   ANTIBIOTICS

Ampicillin, streptomycin sulphate, chloramphenicol, tetracycline, rifampacin and spectinomycin were all obtained from Sigma.

Rifampacin was freshly dissolved in a small volume of methanol and added directly to the sterilized medium at 50°C. Tetracycline was dissolved in 50% ethanol stored at -20°C and added directly to sterilized medium. Aqueous solutions of ampicillin, streptomycin sulphate and spectinomycin were prepared, stored at 4°C and added directly to sterilized medium at 50°C. Chloramphenicol

and erythromycin were dissolved in 95% ethanol and stored at -20°C until use. When necessary, the level of antibiotic to be used was determined by calculating the minimum inhibitory concentration for the particular strain. Alternatively concentrations recorded in the literature were used.

M. 5.   ENZYMES

Restriction and ligation enzymes were obtained from BRL. Calf intestine Alkaline phosphatase was obtained from Boehringer Mannheim.

M. 6.   TRIPHOSPHATES

dGTP, dCTP, dTTP were sodium salts by Boehringer and were diluted in H_2O to a final concentration of 10mM. ^{32}p (dATP) was obtained from Amersham. A stock of 250 uCi with a specific activity of 800 Ci/mMole was obtained as required.

M. 7.   ISOLATION OF ANTIBIOTIC RESISTANT MUTANTS

A late log phase culture (10 ml) was pelletted by centrifugation at 5000g for 10 min. The cells were resuspended in TES Buffer then pelletted again. The

pellet was resuspended in 1 ml TES Buffer and 5 aliquots of 0.1 ml plated on agar plates incorporating the appropriate antibiotic. Frequencies of mutations were estimated by serial dilution of the remaining 0.5 ml and plating 0.1 ml of the higher dilutions on medium agar plates without the antibiotic. The plates were incubated until resistant colonies appeared. Colonies were purified by re-streaking on appropriate selective plates. Isolates were checked using gram stain, catalase reaction and scoring for auxotrophic or other resistance markers if appropriate.

M. 8.   TRANSFORMATION OF *E. coli*

The strains routinely transformed were *E. coli* C600, *E. coli* S17-1 and *E. coli* JA221.

Preparation of competent cells:

An overnight culture was diluted 1 in 10 ml LB and grown to O.D. A_{600} 0.4-0.6. The cells were chilled on ice and centrifuged at 4000g for 10 min at $4^{0}C$. The pellet was resuspended in 5 ml of ice cold 0.1 M $MgCl_2$ and transferred to a pre-chilled 10 ml centrifuge tube. The cells were centrifuged for 5 min at 4000g. The pellet was resuspended in 5 ml ice cold 50 mM $CaCl_2$ and kept on ice for 45 min. The suspension was again centrifuged for 5 min and the cells resuspended in 1 ml of ice cold 50 mm $CaCl_2$. At this stage the cells were considered competent.

Uptake of plasmid DNA:

DNA to be transformed was dissolved in 20 ul of TE buffer and added to 0.2 ml cold competent cells. The transformation mix was held on ice for 30 min then transferred to a $42^{\circ}C$ waterbath for a 2 min heat shock. LB (0.7 ml) was then added and growth for 30 min at $37^{\circ}C$ permitted. The cells were centrifuged at 4000g for 3 min and resuspended in the 50 ul of LB remaining when the supernatant had been poured off. This 50 ul was plated on selective medium. Transformants appeared after overnight incubation at $37^{\circ}C$. DNA free competent cells were also plated routinely as a control.

M. 9.   TRANSFORMATION OF *B. subtilis* 168 MetA

Preparation of Competent Cells:

500 mls of SP1 medium in a 1 litre bottled flask was prepared. 5 mls of the SP1 medium was added to a 50 ml flask and inoculated with 100 ul of glycerol stock of *B. subtilis* 160 MetA. This was incubated overnight at $30^{\circ}C$ shaking vigorously (>200rpm) or at $37^{\circ}C$ shaking slowly (<150 rpm). 45 mls SP1 from the 1 litre flask was removed to a 250 ml flask and the overnight culture transferred to this. This was set shaking vigorously at $37^{\circ}C$ for 4 hours 50 mins (i.e. until the cells had reached late log phase). The 50 ml of late log phase culture was added to the 450 mls SP1 remaining in the 1 litre flask. It was very important to maintain aeration by shaking continuously. To the 500 mls of culture 12.5 ml 0.1 M $MgCl_2$ and 5 ml 0.05 M $CaCl_2$ were added.

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Slow shaking was continued at 37°C for a further 90 mins. Cells were harvested immediately by centrifugation at 10,000 rpm for 10 mins at 5°C. Cells were resuspended in 50 ml of SP-2 containing 10% glycerol. One ml aliquots were distributed in eppendorf tubes, quick freezed in freezing ethanol and stored at -80°C until needed for transformation.

Transformation of Competent Cells:

One ml of transformation medium (Tm) was warmed to 37°C in a plastic 10 ml tube. DNA samples for transformation, including controls, were added to sterile plastic 10 ml tubes (1 ug per tube). Competent cells were removed from -80°C and thawed at 37°C. One ml of thawed cells was added to 1 ml of warmed Tm in the 10 ml tube. This was gently agitated at 37°C for 5 mins. 100 μl of this suspension (Tm plus cells) was added to each DNA sample in 10 ml tubes and agitated well at 37°C for 30 mins. To allow phenotypic expression of antibiotic markers the transformation mixture was supplemented with an equal volume of LB broth followed by shaking at 37°C for 45 mins. Plating on appropriate selection agar media was then performed. Transformants appeared after overnight incubation.

M. 10.   GENERATION OF PROTOPLASTS OF *S. lactis* 712
(MG1363/MG3020)

An overnight culture (inoculated from stocks) in GM17 broth provided a 2% inoculum for 50 mls GM17 which was allowed to grow, at maximum, to an OD

600 nm of 0.5. For each transformation planned 10 mls was pelletted in 50 ml sorvall tubes by centrifugation at 6,000 rpm for 5 mins. This pellet was resuspended in a 10 mg/ml solution of lysozyme made up in SGM17 protoplast buffer (10 mls, filter sterilized). This solution was incubated at 37°C and monitored for protoplast formation by phase contrast microscopy. With *S. lactis* 712 strains, 15 mins. incubation using this procedure has proved optimum regarding transformation efficiency, if protoplasts are for use in protoplast transformation (Ciaran Geoghegan, personal communication). Protoplasts were then pelletted gently (4,000 rpm/10 mins) and washed twice in 10 mls of an isotonic buffer (SGM17 protoplast buffer) followed by gentle resuspension of 1 ml of the same buffer. This protoplast suspension was stored on ice. The percentage of protoplasted to non-protoplasted cells was determined by diluting in SGM17 protoplast buffer followed by plating on SGM17 agar and GM17 agar. Only non-protoplasted cells are supported by GM17 agar. Percentage of protoplasts was routinely 99.9% using this procedure.

M. 11.   TRANSFORMATION OF *S. lactis* MG1363 PROTOPLASTS

Protoplasts were prepared in the manner described in M. 10.

The protoplast suspension was then washed in SMM-Ca^{+2} buffer and resuspended in 1 ml of SMM-Ca^{+2} buffer. Plasmid DNA for transformation (concentrated in 10μl maximum) was placed in a 1.5 ml Eppendorf tube with an equal volume of 2 xSMM-Ca^{+2} buffer. 100 μl of protoplast suspension was added to this followed by 300 μl of 35% PEG (6,000 in SMM Ca^{+2}), mixed gently by inversion and incubated at room temperature for 20 minutes. The

PEG was then diluted using SMM-Ca^{+2} buffer, the cells were harvested by centrifugation in an Eppendorf centrifuge, washed once in SMM-Ca^{+2} buffer, and resuspended in 1 ml of SGM17 broth and allowed to incubate for 1 h at room temperature to allow phenotypic expression. The transformation mix was plated on selection plates containing the appropriate antibiotic, using the soft agar overlay technique, and the appearance of transformants was scored after 2 days incubation at 30°C. Overlay consisted of 3 mls SGM17 solidified with 0.7% agar. Transformation mix (0.5 mls) was mixed with this and spread onto SGM17 plates solidified with 1.5% agar. Antibiotic concentrations used were the minimum to inhibit growth of whole cells (e.g. 4ug/ml Cm).

M. 12.   CONJUGATION BETWEEN _E. coli_ STRAINS

Conjugation between _E. coli_ was facilitated by the use of antibiotic resistant strains which could be used for counter-selection of recipients. 2 x 0.7 ml quantities of donor and 2 x 0.7 ml of recipient were pelletted in eppendorf tubes, washed once with LB and each resuspended in 0.7 mls of LB. One donor and one recipient tube were pooled to give the mating mix. The mating mix, plus the individual suspensions of donor and recipient were pelletted and resuspended in 100 _u_l of LB. These suspensions were pipetted onto 0.22 _u_m filters placed on LB plates to give three cultures; the mating, recipient control and donor control. After incubation overnight at 37°C the filters were removed and placed individually in 4 ml quantities of 0.85% saline. Cultures were resuspended, diluted as appropriate and plated on selective agar media. Selection was for the plasmid being transferred and the recipient strain. Donor and recipient were plated as controls. Plates were incubated at 37°C and transconjugants appeared overnight. Frequencies of transfer were expressed as transconjugants per recipient. (Number of recipient cells used was as determined for the mating mix, using suitable selection against the donor strain.)

M. 13   CONJUGATION BETWEEN _E. coli_ AND
             GRAM POSITIVE STRAINS

(a)   _E. coli S17-1 (pAM401-mob) X S. lactis MG1363 protoplasts_:

S. lactis MG1363 protoplasts were generated as described in M. 10. Two aliquots of protoplasts were pelletted and each resuspended in 0.7 mls of SGM17

protoplast buffer in Eppendorf tubes (recipients). Two aliquots of 0.7 mls late log phase *E. coli* donor cells were also pelletted, washed and resuspended in 0.7 mls of SGM17 protoplast buffer (donors). Such treatment resulted in approximately equal concentrations of donor and recipient cells. 0.7 mls each of donor and recipient were mixed (mating mix), pelletted and resuspended in 100ul SGM17 protoplast buffer. Separate tubes of donor and recipient cells were treated similarly. These suspensions were transferred by pipette onto three 0.22 um filter placed on separate SGM17 agar plates: the mating, the donor control and recipient control. Plates were incubated for 18 hours. Filters were then tarnsferred to 2 mls of SGM17 protoplast buffer and cultures resuspended. 100ul quantities of each were plated onto SGM17 agar containing nalidixic acid 5ug/ml and chloramphenicol 4ug/ml to select for transconjugants. Incubation at 30°C for 2 to 4 days was required before transconjugant colonies became visible. After appropriate dilutions of mating mix recipient cell numbers were determined on SGM17 agar containing 5ug/ml nalidixic acid. Transfer frequency was expressed as transconjugants per recipient.

(b) Transfer from *E. coli* S17-1 (pAM401-mob) to *S. lactis* whole
     cells and to other gram positive species:

The mating procedure was essentially the same as that for *S. lactis* protoplasts except that whole cells were used. Late log phase cultures of donor and recipients were always used, 0.7 mls of each, except where otherwise indicated. 0.85% saline was always used for washings and resuspensions.

In matings to *S. lactis* MG1363 and MG3020 overnight incubation of the mating was on BHI agar and selection was carried out on GM17 containing Nalidixic

acid (50*u*g/ml), Cm (5*u*g/ml).   Em (30*u*g/ml) was also included when selecting for MG3020.   Conditions used in attempted matings to other strains are recorded in the Results section.

M. 14.   FILTER MATING PROCEDURE USED FOR TRANSFER OF pAMB1 FROM *S. lactis* TO VARIOUS RECIPIENTS

Overnight cultures of *B. subtilis, B. thuringiensis, S. faecalis* in BHI broth (5 mls), *S. lactis* in GM17 (5 mls) and mid exponential phase cultures of *C. acetobutylicum* in TYG medium (8 mls) were centrifuged and the bacterial pellets resuspended in 2.5 mls holding buffer (HB).   Mating mixtures were prepared by combining, in pairs, the suspensions of bacteria in HB.   Harvesting of mating mix was recipient dependent.   For *S. lactis* and *B. subtilis* mating mix was centrifuged, pellet resuspended in minimal volume (approx. 0.2 ml) and transferred by pipette to a membrane filter (0.2 *u*m pore size) on RCM agar. With *B. thuringiensis* and *S. faecalis* the mating mixture was transferred to a similar filter by vaccuum filtration and then placed, bacteria uppermost, on RCM agar.   *C. acetobutylicum* was treated similarly but incubation was bacteria lowermost on RCM agar.   Manipulations with *C. acetobutylicum* were not carried out anaerobically.

After incubation of the mating mixtures at 30°C for 24 hours (matings with *C. acetobutylicum* anaerobically in Oxoid anaerobic jar) the bacteria were recovered by resuspending in 5 ml HB.   In the case of *C. acetobutylicum* resuspension was by washing bacteria from petri dish.   Suspensions were centrifugred and resuspended in 1 ml of HB.   The numbers of recipient bacteria were assessed

by serial dilution in HB and the plating of samples on appropriate selective media, as indicated in R . (Em was omitted for recipients). Transconjugants carrying pAMB1 were selected by plating on the selective medium appropriate for the recipient to which Em had been added.

Frequency of transfer of pAMB1 was expressed as the number of transconjugants per recipient cell.

M._15.   MINI-PLASMID PREPARATION FROM *E. coli*

RAPID BOILING PREPARATION (Holmes and Quigley, 1981):

A patch of growth from a fresh plate culture or the pellet from 1 ml of fresh liquid culture was suspended in 300 μl of STET buffer in a microfuge tube. Freshly prepared lysozyme (20 μl of 10mg/ml) was added and the tube left at room temperature for 10 mins. The tube was placed in boiling water for 60 sec and then immediately removed to a microfuge for a 10 min centrifugation. The supernatant was removed to a new tube and the plasmid DNA precipitated by the addition of an equal volume of isopropanol. The tube was placed in -20°C for 20 min and then centrifuged for 5 min. The isopropanol was removed by an ether wash and the pellet dried by vacuum. The DNA was dissolved in 50μl of TE containing RNAase (1 unit/ml). 10μl was more than sufficient for analysis by agarose gel electrophoresis.

M. 16.   PREPARATION OF BULK PLASMID DNA FROM *E. coli*

A 5 ml overnight culture (inoculated from stock) was used to inoculate 250 ml
LB broth to 1-2%. This was incubated overnight at 37°C. Cells were pelletted
by centrifugation in Sorvall centrifuge, 10,000 rpm for 10 mins. Pellet was
resuspended in 2 ml 25% sucrose, 50mM Tris (pH 8.0). The suspension was
transferred to 50 ml Sorvall tube. 0.4 ml of lysozyme (20 mg/ml in 0.25 M
Tris, pH 8.0) was added and incubated on ice for 5 mins. 0.8 ml EDTA (0.25
M, pH 8.0) was added and the solution was held on ice for a further 10 mins.
Lysis was initiated by addition of 3.2 ml Triton-Mix and complete lysis had
occurred after 15 mins on ice. Lysate was cleared by centrifugation at 15,000
rpm for 30 mins. Supernatant containing plasmid DNA was removed to a
sterile container and stored on ice. Plasmid DNA was purified by CsCl gradient
ultra centrifugation.

M. 17.   PLASMID ISOLATION FROM *S. lactis*

The method used routinely to isolate plasmid DNA from lactic streptococci has
been described by Anderson and McKay (1983). This could be used for both
large scale preparation and isolation of plasmid DNA and "mini preparations" to
screen strains for the presence of plasmid DNA. The protocol is described
below and the volumes in brackets refer to those used in the large scale method.

Cells were grown to late log phase in GM17 broth using volumes of 3 ml (600
ml), harvested by centrifugation at 9,000 rpm for 10 min and resuspended in

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379 μl (7.5 ml) of 6.7% sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0. The suspension was then warmed to 37°C, 96.5 μl (7.5 ml) of lysosyme (10 mg/ml in 25 mM Tris, pH 8.0) was added after incubation at 37°C for 5 min, 48.2 μl (3.75 ml) of 0.25 M Tris, pH 8.0 was added and mixed by inversion. 27.5 μl (2.25 ml) of SDS (20% w/v in 50 mM Tris, 20 mM EDTA, pH 8.0) was added and mixed immediately by swirling and incubated at 37°C for 5 to 10 minutes. The suspension was vortexed at the highest setting for 30 seconds. 27.6 μl (2.4 ml) of freshly prepared 3.0 N NaOH was added and mixed thoroughly by inversion for 10 minutes. 49.6 μl (3.9 ml) of 2 M Tris-HCl (pH 7.0) was added and mixed gently for 3 minutes. To this, 71.7 μl (5.7 ml) of 5 M NaCl was added and thoroughly mixed. 700 μl (55.8 ml) of phenol, saturated with 3% NaCl, was then added, mixed well and centrifuged for 5 minutes. The upper phase was removed and extracted with 700 μl (55.8 ml) of chloroform: isoamyl alcohol (24:1) and centrifuged for 5 minutes. Once again the uppper phase was removed and the DNA percipitated with 1 volume of isopropanol. This solution was then held at -20°C overnight.

The suspension was then centrifuged for 10 minutes and the isopropanol discarded. The pellet was resuspended in 20μl of TE for small scale DNA.

M. 18.   ISOLATION OF LARGE PLASMID DNA FROM *S. lactis*

Cells were grown and lysed as in M. 17. using large-scale volumes. Vortexing step was omitted. Lysate was held in 250 ml Sorvall tube and pH slowly adjusted to 12.4 by adding 3 M NaOH dropwise and stirring with plastic pipette. Following incubation at room temperature for 20 mins the pH was adjusted to

8.6 by addition of 2 M Tris-HCl pH 7.0. pH was monitored throughout this step. Volume was accurately measured and solution was removed to a 50 ml centrifuge tube. This was made 1 M with respect to NaCl by addition of 0.25 volumes of 5 M NaCl. After mixing and incubation on ice for 4 hours the solution was cleared by centrifugation at 13,000 g for 30 mins at 4°C. Supernatant was decanted, volume measured and 0.25 volumes of 50% PEG 8,000 added. After thorough mixing this solution was incubated overnight on ice. Centrifugation at 13,000 g for 30 mins at 4°C gave a plasmid pellet. This was washed with 10 ml of TE and resuspended in 1.5 ml of TE. To purify DNA this volume was brought up to 6 ml maximum with similar samples or TE and purified on a CsCl gradient.

This procedure was used for preparing pAMB1 DNA.

M. 19. PREPARATION OF TOTAL DNA FROM GRAM-NEGATIVE AND GRAM-POSITIVE STRAINS

1.5 ml of an overnight culture was pelletted in an eppendorf tube and resuspended in 500ul of 20% sucrose, 50 mM Tris, 1 mM EDTA (pH 8.0). 100 ul of 10 mg/ml lysozyme in 25 mM Tris (pH 8.0) was added and after incubation at 37°C for 10 mins 60ul of 0.25 m Tris, 50 mM EDTA (ph 8.0) was added. 40 ul of SDS (20% w/v in 50 mM Tris, 20 mM EDTA, pH 8.0) was added and mixed immediately by swirling. This was incubated at room temperature until solution became clear. A phenol extraction was immediately carried out followed by an extraction in Kirly mix and one with Chloroform-isoamylalcohol. DNA was precipitated by addition of 1 ml of

isopropanol, by centrifugation.   Pellet was dried and resuspended in 50 *u*l of
sterile water. This   procedure   could   be   used   for   *E. coli*,   *B. subtilis*,   *B.*
thuringensis,   *S. faecalis*   and   *S. lactis*.

M. 20.   PURIFICATION OF DNA USING CAESIUM CHLORIDE (CsCl) DENSITY GRADIENT CENTRIFUGATION

The DNA solution (maximum of 6 mls) was added to 6.9 g CsCl in a sterile
universal and the CsCl gently dissolved.   0.18 mls of Ethidium Bromide (EtBr)
(10 mg/ml in sterile water) was added.   This solution was added to heat seal
ultracentrifuge tubes and total solution weight was brought up to 14.1 g using
remaining plasmid solution or 10 mM EDTA.   Tubes were filled with mineral
oil and heat sealed.   Centrifugation was carried out for at least 22 hours at
50,000 rpm in a Beckman ultracentrifuge.   DNA bands were visualized using
ultraviolet light and drawn off using 0.9 mm bore needle and 1 ml syringe.
EtBr was removed by repeated extractions with isopropanol saturated with 20 x
SSC.   CsCl was removed by dialysis against TE employing at least four buffer
changes.   DNA concentration was estimated by mesuring OD at 260 mm.   DNA
stocks were stored at 4^{0}C.   Plasmid or chromosomal DNA could be purified
using this procedure.

M. 21.   TRANSFER OF DNA TO NITROCELLULOSE FILTERS
BY SOUTHERN BLOTTING:

Restricted total DNA was analysed in a 1% agarose gel overnight, stained and photographed before blotting. A ruler was placed along one side of the gel to facilitate measurement of distance when comparing the photograph with the final autoradiograph.   Before denaturation of the gel, it was necessary to nick the larged DNA modecules by immersion in 0.25 M HCl.   The gel was washed with tap water and then immersed in denaturing solution and placed on a shaking table at room temperature for 40 min.   The denaturing solution was poured off and the gel washed in tap water again.   The gel was placed in neutralizing solution on the shaking table and agitated for 1 h at room temperature or until the surface of the gel was below pH 8.5 (as indicated by pH paper).   The gel was then transferred to an upturned gel base on which there was a sheet of 3 x Whatman paper which had been soaked in 20 x SSC. This paper sheet hung over the gel base at either end and dipped into a bath of 10 x SSC.   Nitrocellulose paper cut to the exact size of the gel was pre-soaked in 2 x SSC until fully wetted and was then placed on top of the gel with care taken to remove any air bubbles.   The well positions were marked on the nitrocellulose for later identification.   Two pieces of 3 x Whatman paper soaked in 2 x SSC cut to the same size as the gel were placed on top of the nitrocellulose filter.   Finally, paper towels about 10 cm high were put on top and compressed lightly with a piece of glass.   The presence of cling-film around the gel ensured that the only contact between the underlying Whatman paper wick and the paper towels was through the gel.   After overnight blotting the nitrocellulose was removed from the gel and soaked in 6 x SSC for 5 min then

blotted dry between two pieces of filter paper. The filter was then baked for 2 h at 80°C. The gel was restrained to check for the full transfer of DNA to the nitrocellulose.

<u>M. 22.</u>   <u>NICK TRANSLATION</u>

The nick translation reaction was set up by the addition of the following to a microfuge tube:

10 x Nick Translation buffer	4 ul
DNA	10 ul
dGTP (10 mM)	1 ul
dCTP (10 mM)	1 ul
dTTP (10 mM)	1 ul
(^{32}p) dATP (800Ci/mM)	1 ul
DNAase (0.1 ug/ml)	0.5 ul
Polymerase 1	1 ul

The DNAase was the last component of the reaction to be added. The reaction was allowed to proceed for 60 min at 16°C. Separation of labelled DNA was made on a Sephadex G-50 column (Maniatis et al., 1982). The Sephadex was equilibrated with TES buffer and poured into a disposable syringe which had been plugged with glass wool.

DNA/DNA HYBRIDIZATION

The filter into which DNA had been blotted by Southern transfer was soaked in 6 x SSC for 2 min. The filter was then pre-hydridized at $68^{\circ}C$ in an air-tight plastic box containing pre-hybridization fluid (0.2 ml per cm^2 of filter). The pre-hybridization fluid consisted of:

6 x SSC

0.5% SDS

5% Denhardt's Solution

100 ug/ml denatured salmon sperm DNA

After 4 h the pre-hybridization fluid was removed and replaced by 25 ml of hybridization fluid. The hybridization fluid was the same as the pre-hybridization fluid except that it contained 0.01 M EDTA. The DNA probe was denatured by boiling for 5 min in a microfuge tube and was then added to the hybridization mix. The air-tight box containing the hybridization mix was re-incubated for 16 h at $68^{\circ}C$. The filter was then removed and the hybridization mix stored for re-use or disposed of as radio-active waste. The filter was submerged in a tray containing a solution of 2 x SSC and 0.5% SDS at room temperature, where it was washed for 5 min. It was then transferred to a fresh tray containing 2 x SSC and 0.1% SDS where it was soaked for 15 min at room temperature. The filter was removed again to a plastic box containing a solution of 0.1 X SSC and 0.5% SDS. The box was incubated for 2 h at $68^{\circ}C$ for homologous hybridization ($51^{\circ}C$ for non-homologous hybridization). After 2 h the buffer was changed and incubation carried on for a further 30 min. The filter was dried at room temperature, sealed in a heat seal

bag and exposed to x-ray film in the dark at room temperature. The required exposure time varied from 4 days to 2 weeks. The autoradiograph was developed by a 5 min immersion in developer followed by two 1 min washes in fixer.

M. 24.   CLEAVAGE OF DNA BY RESTRICTION ENDONUCLEASES

Reactions were carried out in the appropriate buffer for each enzyme in a microfuge tube. The DNA to be cleaved was dissolved in TE buffer. An appropriate volume of concentrated restriction buffer was added to bring the overall buffer concentration to 1 X. The reaction volume was always large enough to ensure at least a 10 fold dilution of added enzyme. Reactions were held at the appropriate temperature for the particular enzyme (depending on manufacturers specifications) which was $37^{O}C$ for most enzymes. Reactions were allowed to proceed for 1 h and were then stopped by a temperature shift to $65^{O}C$ for 10 min. Fragments were separated on a 0.7% agarose-Tris acetate gel. Gels were stained in distilled water containing ethidium bromide (10 ug/ml). Fragment visualization and photography were as described previously for plasmid bands.

M. 25.   DEPHOSPHORYLATION OF VECTOR DNA

Restricted plasmid DNA was ethanol precipitated and washed twice in 70% ethanol. The DNA pellet was dried and resuspended in 50 ul of sterile water.

Dephosphorylation reactions were carried out in a total volume of a 100 ul with 10 ul CIP buffer (10 X) and 1 ul of calf intestinal phosphatase enzyme. The reaction was allowed to proceed for 30 min at 37°C, when a further 1 ul of enzyme was added. To stop the reaction 20 ul of EDTA (100 mM) were added along with 60 ul of H_2O. The tube was held at 68°C for 15 min. The solution was extracted twice with phenol and then with ether. The DNA was precipitated by adding 20 ul of 3 M sodium acetate and 400 ul of ethanol and leaving at -20°C for 1 h.

M. 26.   LIGATION OF DNA FRAGMENTS

After the restriction of target and vector DNA, the restriction enzymes was denatured by heating to 65°C for 10 min. An equal volume of Kirby mix was added and the two phases mixed well. The tubes were centrifuged to separate the two phases and the aqueous layer was transferred to a new tube where the remaining Kirby mix was removed by an ether extraction. The solution was made 0.3 M with respect to sodium acetate and 2 volumes of 95% ethanol were added. The tube was placed at -20°C for 20 min then centrifuged at 26,000g for 15 min. The pellet was washed in 70% ethanol and then dried under vacuum. The DNA was resuspended in TE buffer. (DNA which had been dephosphorylated was resuspended in TE buffer also). The vector and target DNAs were mixed in the desired ratios in a volume of 17 ul. The solution was heated to 55°C for 5 min to linearize any recirculized DNA, then placed on ice. Ligation buffer (2 ul of 10 X) and 5 units of T4 ligase were added. The total volume was 20 ul. The reaction was incubated at 16°C for 16 h. Immediately before transformation the DNA was heated to 55°C to separate any

cohesive ends that had not ligated. Ligation mix was diluted at least five times before transformation (to 100ul). 20 ul aliquots were used in transformation of competent *E. coli..*

R. 1. TRANSFORMATION OF *Streptococcus lactis* and *Bacillus subtilis*

Transformation of *S. lactis* MG1363 protoplasts was carried out using pAM401 plasmid DNA. Transformation was recorded at a frequency of 1×10^3 transformants per *u*g of DNA when carried out at AFT, Moorepark. However, several attempts to use the same procedure at NIHE were not successful. This reflects literature reports of poor reproducibility obtained in transformation of *S. lactis* protoplasts.

Transformation of competent *B. subtilis* 168 was carried out with three plasmids. pAM401, pIL253 and pAMB1. Frequencies of transformation are recorded on Table 2. DNA prepared from transformants was analysed by agarose gel electrophoresis and results presented on Plate 1.

TABLE 2   Transformation Frequencies to *B. subtilis*

Plasmid	Size	Frequency of Transformation (CFU/ug DNA)
pAM401	10.4	1×10^6
pIL253	4.8	1×10^5
pAMB1	26.5	1×10^3

PLATE 1: Screening of *B. subtilis* transformants for the
presence of plasmid DNA.

1. pAM401 DNA (control)

2. pAM401 transformant

3. pIL253 transformant

4. pAMβ1 transformant

5. pAMβ1 transformant

6. pAMβ1 transformant

7. pAMβ1 transformant

8. pAMβ1 transformant

9. pAMβ1 transformant

10. pAMβ1 DNA (control)
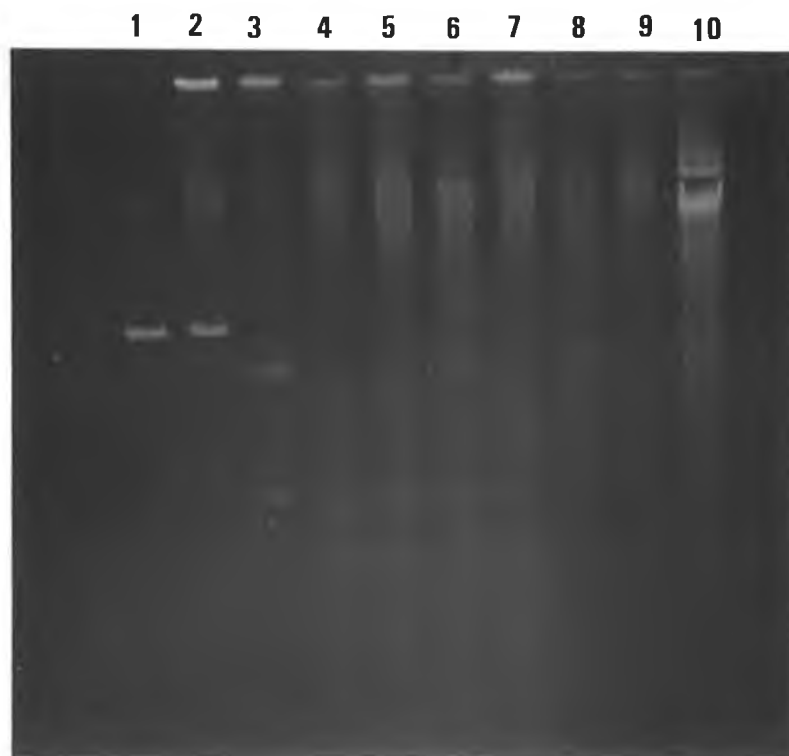
R.2.   TRANSFER OF   pAMβ1 TO VARIOUS GRAM-POSITIVE STRAINS
BY CONJUGATION

Conjugal transfer of pAMβ1 has been reported to several different species usually at low frequencies using a variety of methods.   Originally isolated from *S. faecalis* it's host range has been shown to include a number of other *Streptococcus* species, various *Lactobacillus* species, *Staphylococcus aureus*, various species of *Bacillus*, including *B. subtilis*, and *Clostridium acetobutylicum*. In this work it was attempted to simplify and standardize the conjugation protocol for a number of strains and to examine the frequencies of conjugation in our laboratory compared to those reported.   In gram-negative genetics, conjugation has proved a very useful tool because of the high transfer frequencies obtained and also because of the ease of carrying out several matings conveniently and quickly.

The procedure adopted is included in Methods (M. 27).   This procedure was not suitable for all strains tested during repeated attempts to transfer pAMβ1. Modifications required are documented in M. 27.   Transfer frequencies for strains tested are reported on Table 3.   Physical evidence for presence of pAMβ1 was obtained by hybridization studies with p^{32} labelled pAMβ1 (Plate 2).

TABLE 3   Frequency of Transfer of pAMβ1 by Conjugation:

Donor (*S. lactis*)	Recipient	Ab. Selection [c] (ug/ml)	Transfer Frequency [b]
MG3020	*S. lactis* LM0230 Rif	Rif, 400; Em, 30	9×10^{-5}
MG3020	*B. subtilis* 168 Met A	Minimal medium [a] Em, 15	7.5×10^{-6}
LM0230	*S. lactis* MG1363	Sm, 600; Em, 30	1×10^{-4}
MG3020	*B. thuringensis* (NIHE, Sp)	Sp, 600; Em 15	1.5×10^{-7}
MG3020	*S. faecalis* (NIHE, Rif)	Rif, 400; Em, 20	2.5×10^{-7}
MG3020	*C. acetobutylicum* SA3	Minimal [a] Medium Em 30	2.1×10^{-5}

(a)   Minimal media for *B. subtilis* and *C. acetobutylicum* which do not support growth of *S. lactis* are listed in Methods (M. 2).

(b)   Transfer frequency expressed as transconjugant per recipient.

(c)   Frequencies of spontaneous chromosomal resistance for these strains is reported on Table 4.

TABLE 4   Frequencies of Spontaneous Mutation to Antibiotic
Resistance:

Frequency of Spontaneous Mutation [a]

Strain	EM	Rif	Sp	Sm
S. lactis				
MG3020	R[b]	$<10^{-8}$	ND	R
MG1363	$<10^{-9}$	$<10^{-8}$	ND	R
S. lactis				
LM0230	$<10^{-9}$	10^{-8}	ND	
B. subtilis				
168	$<10^{-9}$	ND	ND	ND
B. thuringensis	$<10^{-9}$	ND	2×10^{-9}	ND
S. faecalis	$<10^{-9}$	4×10^{-9}	3×10^{-7}	ND
C. acetobutylicum	$<10^{-9}$	ND	ND	ND

(a)   Antibiotic concentrations as in Table 3 for each strain.

(b)   R => strain resistant to this antibiotic.

(c)   ND => not carried out.

PLATE 2: Hybridisation of *BstEII* restricted pAMβ1 transformant and *BstEII* restricted transconjugant DNA with ^{32}P labelled pAMβ1.

1. *B. subtilis* 168

2. *B. thuringensis* (pAMβ1)

3. *B. subtilis* 168 (pAMβ1)   ⎤
                                     ⎥ — transformants
4. *B. subtilis* 168 (pAMβ1)   ⎦

5. *B. subtilis* 168 (pAMβ1)   ⎤
                                     ⎥ — transconjugants
6. *B. subtilis* 168 (pAMβ1)   ⎦

7. *S. lactis* 1363

8. *S. lactis* 1363 (pAMβ1)

9. *S. faecalis* (pAMβ1)

10. *S.lactis* 3020 (positive control)

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Plate 3 shows an autoradiogram from a filter blot hybridization where *Bst*E11 restricted plasmid DNA (pAM401) and total DNA (*S. lactis* (pIP501)) were probed with ^{32}p-labelled pAMB1.
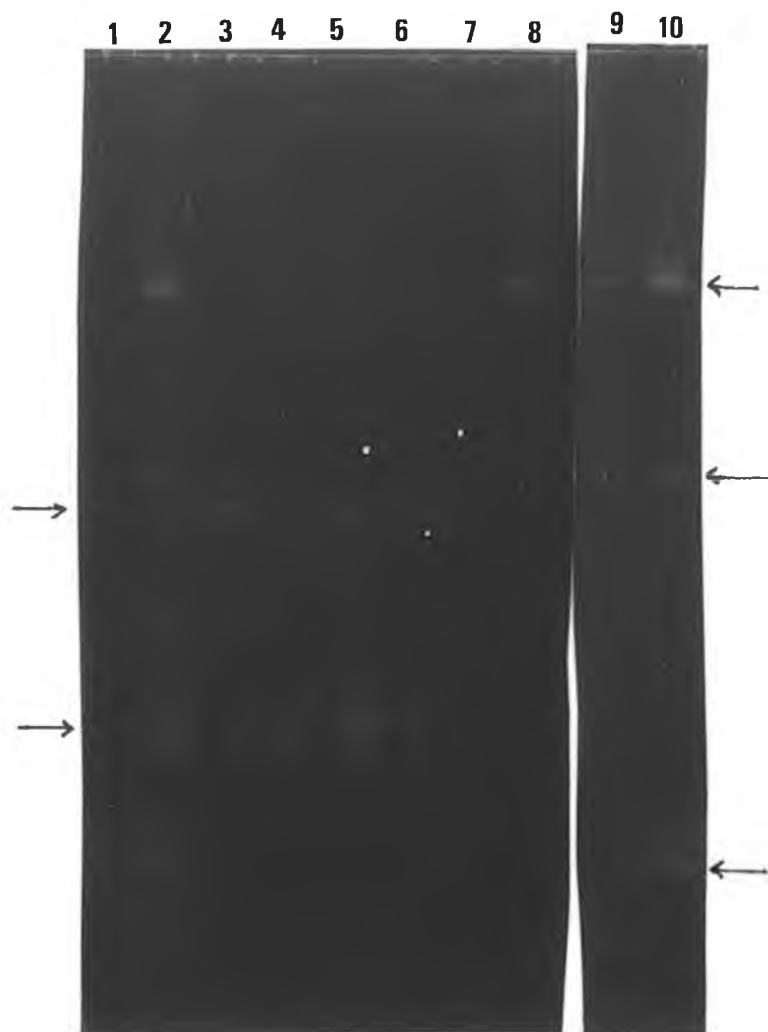
The result suggests homology between pAMB1 and the 10 kb *Bst*E11 fragment from pIP501. The possibility that homology is based on the Em^R resistance regions of the two plasmids is unlikely since the shuttle vector pAM401 (Em^S), which contains a deletion derivative of pIP501 (pGB354) also demonstrates homology with pAMB1. pGB354 has had a 3.6 Kb region containing the Em^R determinant deleted. Hershfield (1979) has suggested that pIP501 and pAMB1 are members of a family of streptococcal plasmids which share related Em^R determinants.

PLATE 3: Hybridisation of pAM401 and pIP501 to ^{32}P labelled pAMβ1.

1.   *S. lactis* (pAM401) *BstEII* restricted total DNA

2.   *S. lactis* (pIP501) *BstEII* retricted total DNA

1   2

pAM401 (10.4 Kbp) →   ← pIP501 (10 Kbp fragment)
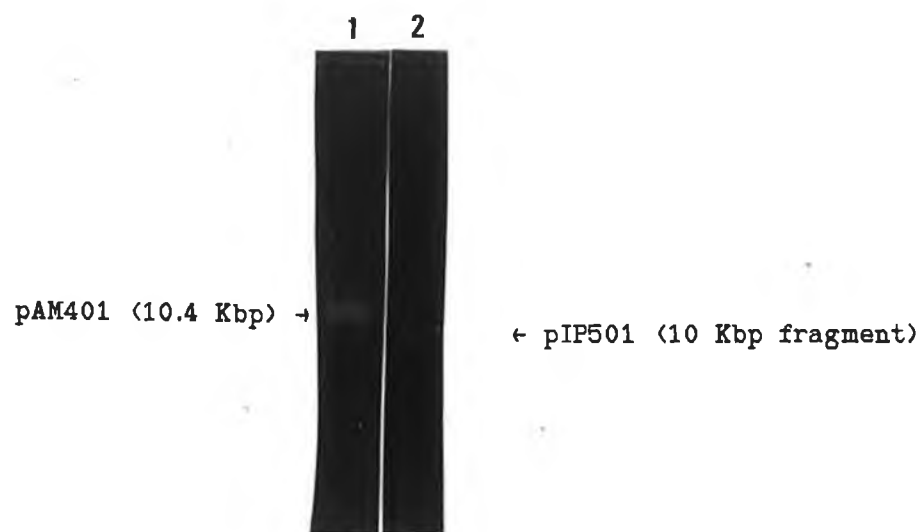
RESULTS - SECTION C

R. 4. IDENTIFICATION OF DELETED FORMS OF pAMB1 IN *B. subtilis*

Previously there have been conflicting reports on stability of pAMB1 in *B.
subtilis* 168 strains following introduction by conjugation. Oultram *et al* (1987)
and Oultram (personal communication) present results supporting the presence of
intact pAMB1 in *B. subtilis*. Van der Lelie & Venema, (1987), discount the
observation of Oultram *et al* observation and present evidence to the contrary.
They observed a 10.6 Kb specific deletion of pAMB1 in *B. subtilis* following
conjugation and protoplast transformation of a restriction minus *B. subtilis*
strain.

Results are presented here which agree with the latter findings. Competent *B.
subtilis* 168 were also transformed with pAMB1, as stability of pAMB1
introduced in this way had not been checked previously.

Plasmid pAMB1 was transferred to *B. subtilis* 168 from *S. lactis* MG3020
(pAMB1) by conjugation and to competent *B. subtilis* 168 (see R. 2.) by
transformation. Total DNA prepared from these strains was restricted with
*BstE*11 and *EcoR*1 and hybridization with p^{32} labelled pAMB1 was carried out.

Plate 5 shows autoradiogarms of the hybridizaitron analysis. Size estimation of
the resulting bands suggest that major deletions have occurred. Based on the
*EcoR*1 restriction evidence (bands equal 4.5 and 6Kb) a deletion totalling 16 Kb
has occurred. The *BstE*11 visable bands total 8.5 Kb (3.3 + 5.0) suggesting a

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PLATE 5: Deleted forms of pAMβ1 in *B. · subtilis* shown by hybridisation with ^{32}P labelled pAMβ1.

1. *B. subtilis* 168 (pAMβ1) transformant (*BstEII*)

2. *B. subtilis* 168 (pAMβ1) transformant (*BstEII*)

3. *B. subtilis* 168 (pAMβ1) transconjugant (*BstEII*)

4. *B. subtilis* 168 (pAMβ1) transconjugant (*BstEII*)

5. *B. subtilis* 168 (pAMβ1) transformant (*EcoRI*

5.0 Kbp →

3.3 Kbp →

← 6.0 Kbp

← 4.5 Kbp
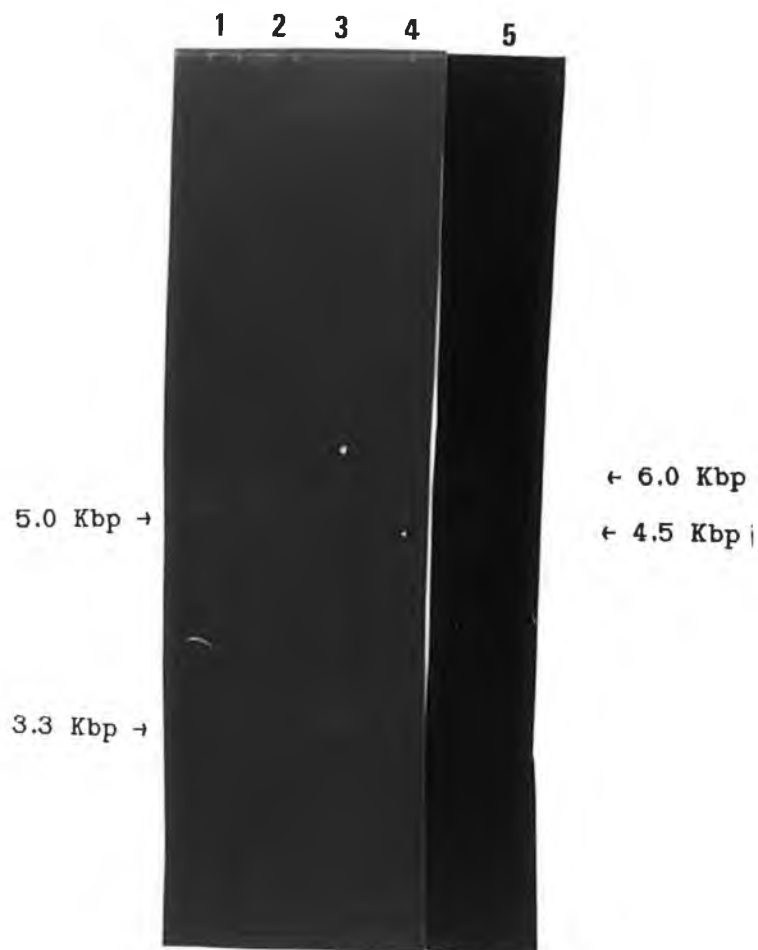
deletion of 18.2 Kb. It is possible that a third, smaller, *BstE*11 fragment exists but was not clear in this experiment. It was difficult to map deletions based on only two restriction patterns, but it appears extensive deletions in the large *EcoR*1 fragment of pAMB1 occurred (see Appendix II). It is apparent from lanes 1 and 2 (transformants) and lanes 3 and 4 (transconjugants) that deletions were specific. These deletions do not compare with deletions reported by Van der Lelie and Venema, 1987. The implicatirons are that deletions do occur in *B. subtilis* but may be strain dependent (a different strain of *B. subtilis* 168 was used in these experiments). Larger deletions may also account for the fact that attempts to mate *B. subtilis* (pAMB1) strains with *S. lactis* and another *B. subtilis* 168 strain were unsuccessful. Van der Lelie and Venema (1987) recorded mobilization of deleted pAMB1 from *B. subtilis* to another *B. subtilis* strain but not to *S. lactis*.

No deletions were observed in other species to which pAMB1 was introduced. Total DNA from *S. faecalis*, *S. lactis* and *B. thuringensis* strains harbouring pAMB1 transconjugants from matings with *S. lactis* (pAMB1) (see R 2 .), was also restricted with *BstE*11 and hybridized with ^{32}p-labelled pAMB1. Results shown on Plate 2 are expected patterns for intact pAMB1 based on published maps (Appendix II).

PLASMID TRANSFER FROM GRAM-NEGATIVE TO GRAM-POSITIVE

BACTERIA BY CONJUGATION

It has been reported recently (Buchanan - Wollaston *et al*, 1987) that the *mob* and ori T mobilization functions of the IncQ broad host range plasmid RSF1010 can mediate the transfer of plasmids from *Agrobacterium* into plant cells as well as transfer between gram-negative bacteria. Buchanan - Wollaston *et al's* findings suggest th*e* presence of a non-specific transfer process, which mediates DNA transfer from a procaryotic to a eucaryotic system and between gram-negative procaryotic cells. Such a system should, therefore, initiate transfer of DNA between the more closely evolutionary related gram-negative and gram-positive eubacteria. A fragment containing the *mob* functions of the broad host range Inc P plasmid RP4 was cloned into the shuttle vector pAM401 and analysed for it's ability to mediate transfer to gram-positive species.

R. 5.   CONSTRUCTION OF pAM401-mob:

The *mob* fragment of RP4 flanked by *Bam*H1 sites was available in pSUP5011 (Simon, 1984)   pSUP5011 was restricted with *Bam*H1 (to release *mob*) and *Eco*R1 (to decrease the likelihood of regeneration of pSUP5011) simultaneously. The *E. coli* - gram-positive shuttle vector pAM401 (Wirth *et al*, 1986) has a single *Bam*H1 site in it's Tc^R gene. The gram negative segment of this vector

is pACYC184 and contains a Cm^R gene. pAM401 was also restricted with *Bam*H1 and ligated with the pSUP5011 restricted DNA. The ligation mix was used to transform competent *E. coli* S17.1 to Cm^R. To screen for insertion of *mob* into the Tc^R gene of pAM401 and to identify transformants containing intact pSUP5011 (also Cm^R) replica plating was carried out on Ap, Tc and Cm respectively. Sensitivity to Ap and Tc indicated transformations containing pAM401::mob. Four such transformants were chosen and further analysed by agarose gel elecrophoresis and mating experiments to another *E. coli* strain.

<u>DNA Analysis</u>:

DNA was recovered from four transformants by the rapid boiling procedure. Because of the similar sizes of pSUP5011 and pAM401-mob, restriction analysis of both plasmids was used to differentiate between them. Plate 6 shows the results of an *Eco*R1, *Bam*H1 double digestion of both plasmids. Lane (2) corresponds to expected bands for pAM401-mob. Lanes (3) and (4) show expected bands for pSUP5011. DNA in lane (1) contains both species, perhaps as a cointegrate or existing simultaneously. The transformant corresponding to DNA appearing in lane (2) contains pAM401 with mob inserted to it's *Bam*H1 site and this strain was designated S17-1 (pAM401-mob) (Figure 1).

<u>R. 6.</u>   MOBILIZATION to *E. Coli* JM83

To further characterise pAM401-*mob*, matings were carried out to *E. coli* strain JM83 which is resistant to Sm 1000ug/ml and allows easy counter selection of the donor strain, S17-1. *E. coli* S17-1 contains the plasmid

PLATE 6: Examination of DNA isolated from transformants for

pAM401-*mob* using *EcoRI* to *BamHI* double digests.

1. Corresponds to pSUP5011::pAM401 cointegrate

2. Corresponds to pAM401-*mob*

3. Corresponds to pSUP5011

4. Corresponds to pSUP5011

5. *EcoRI, BamHI* restricted pSUP5011 (control)

6. Ap[R], Cm[R] transformant corresponds to pSUP5011 minus *mob*

7. *EcoRI, BamHI* restricted pAM401 (control)
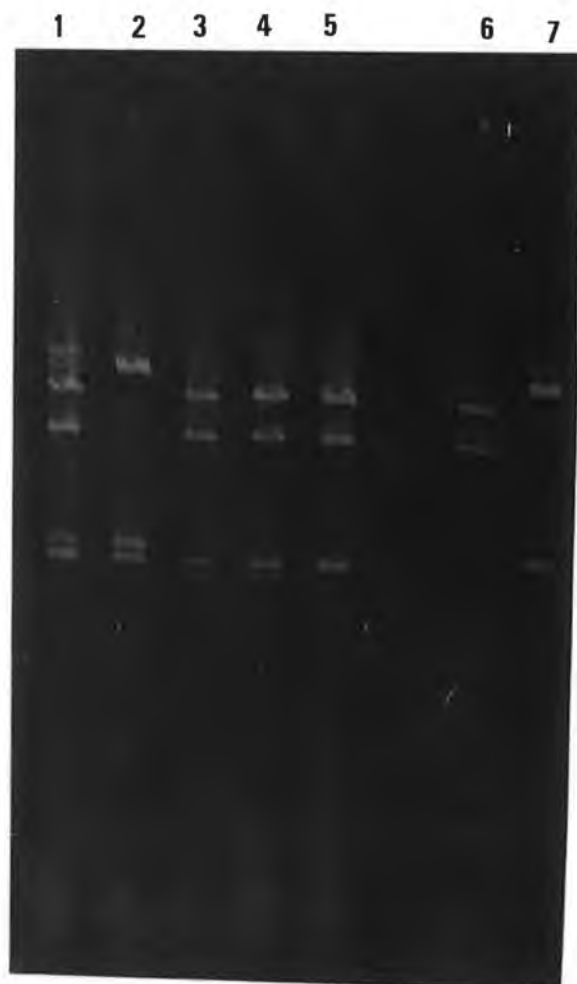
FIGURE 1: Map of pAM401-*mob*
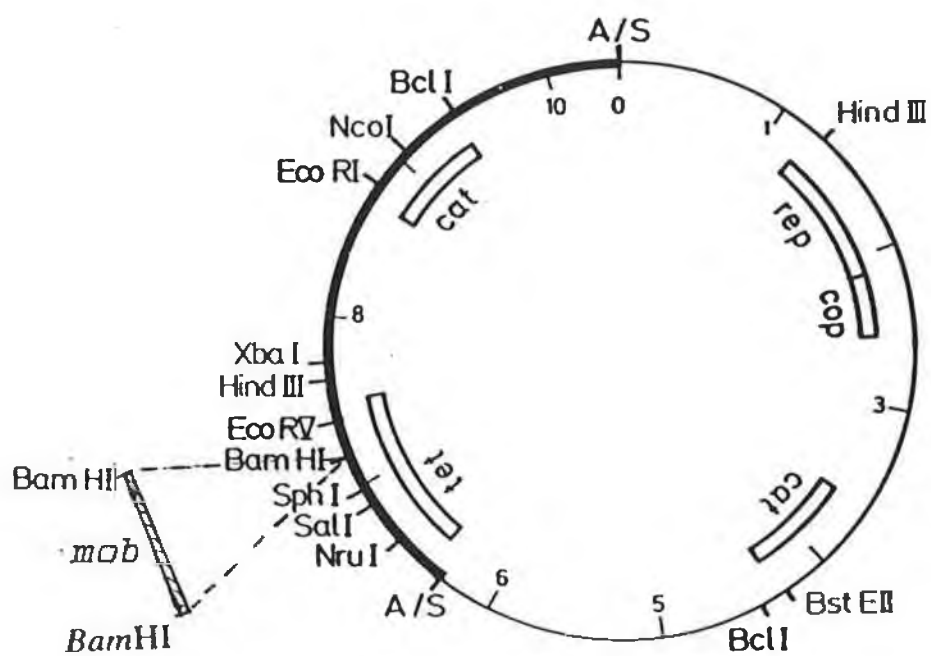
RP4-2-Tc::MuKm::Tn7 integrated into it's chromosome. The integrated plasmid supplies *trans* acting mobilization functions which allow mobilization of any plasmid carrying the oriT (Mob) sequence *in cis*. Matings were also carried out with the donors S17-1 (pSUP5011) and S17-1 (pAM401) as controls. Results are shown in Table 5. There was sufficient evidence that pAM401 contained the RP4 mob and transferred at frequencies similar to pSUP5011 in *E. coli*.

R. 7. MOBILIZATION OF pAM401-mob TO *S. lactis*

To determine if the mob fragment of RP4 could mediate transfer from gram-negative to gram-positive bacteria, in the presence of *trans* acting mobilization functions, a mating experiment between S17-1 (pAM401-mob) and *S. lactis* MG1363 and MG3020 was initiated. Both strains are resistant to Sm 600 ug/ml and it has been determined that *S. lactis* was intrinsically resistant to 50 ug/ml nalidixic acid. Both of these antibiotics could be used to select against S17-1. Results of the matings, with suitable controls included are recorded in Table 6. Matings and selection plates were incubated at $30^{\circ}C$ and transconjugants appeared after 48 hours.

TABLE 5   *E. coli* Matings

Donor	Recipient	Antibiotic[b] Selection (ug/ml)	Transfer [a] Frequency
S17-1 (pAM401-mob)	JM83	Cm^{50};Sm,1000	1.3×10^{-2}
S17-1 (pAM401)	JM83	Cm^{50};Sm,1000	$<1 \times 10^{-8}$
S17-1 (PSup5011)	JM83	Cm^{50};Sm,1000	2.4×10^{-1}

(a) Transfer frequency is expressed as transconjugants recipient and is an average of two experiments.

(b) Spontaneous mutation of JM83 to Cm^R and S17-1 to Sm^R at these antibiotic levels was less than 10^{-8}.

TABLE 6   Matings between *E. coli* and *S. lactis*

Donor	Recipient	Antibiotic Selection [b]	Transfer Frequency [a]
S17-1(pAM401-mob)	MG1363	Sm,Nal,Cm	$1 - 5 \times 10^{-7}$
S17-1 (pAM401-mob)	MG3020	SM,Nal,Cm,Em	$1 - 5 \times 10^{-7}$
S17-1 (pAM401)	MG1363	Sm,Nal,Cm	$<10^{-8}$
DH1 (pAM401-mob)	MG1363	Sm,Nal,Cm	$<10^{-8}$
S17-1	MG1363	Sm,Nal,Cm	$<10^{-8}$

(a) Transfer frequency expressed as transconjugants/recipient.
Result is the frequency range obtained over at least 3
experiments.

(b) Sm, 600; Nal, 50; Cm, 5; Em, 20ug/ml. Usually Nal was used to
select against *E. coli*, Cm to select for plasmid acquisition
and transconjugants were scored for Sm or Em resistance to
verify strain involved. Gram strains and catalase tests were
also used in strain identification.

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Analysis of DNA in pAM401-mob Transconjugants:

As further evidence that *S. lactis* transconjugants contained pAM401-mob and had received the plasmid intact, DNA was prepared from transconjugants isolated from separate experiments, analysed by electrophoresis and also used to transform competent S17-1. Plate 7, lanes 2 to 5 show results of electrophoresis of DNA isolated from *S. lactis* transconjugants. DNA isolation was carried out using the procedure of Anderson & McKay (1984). Similarly prepared *S. lactis* DNA was used to transform competent S17-1. Plasmid DNA can be purified easier from *E. coli* (without significant chromosomal contamination) and clear restriction patterns can be observed by electrophoresis. DNA was prepared from several S17-1 strains transformed to Cm^R with *S. lactis* transconjugant DNA. These were restricted with *Eco*R1 and *Bam*H1 simultaneously and electrophoresed. Results (Plate 9) show the characteristic restriction pattern of pAM401-mob. To determine if transfer ability was maintained matings were carried out to JM83. Transfer frequencies comparable to original S17-1 (pAM401-mob) donor (10^{-2}/recipient) were recorded.

Unrestricted DNA obtained from S17-1 (pAM401-mob) transformants (transformed with plasmid DNA isolated from *S. lactis* transconjugants) (Plate 8) indicate that in transformant number 6, pAM401-mob exists only as a multimer. Restriction analysis (Plate 9) indicates no deletions or rearrangements. This result could be an artifact of transformation and have no relation to conjugation. However, a similar effect was observed in pAM401-*mob* DNA purified from *S. lactis* and *S. faecalis* transconjugants (Plate 7, lanes 5 and 6) where high molecular weight plasmid multimers existed at a high concentration relative to the monomeric forms.

PLATE 7: *S. lactis* and *S. faecalis* transconjugants from matings with *E.coli* S17-1 (pAM401-*mob*)

1. pAM401 DNA (control)

2. *S. lactis* MG3020 (pAM401) (control)

3. *S. lactis* MG3020 (pAM401-*mob*) transconjugant

4. *S. lactis* MG1363 (pAM401-*mob*) transconjugant

5. *S. lactis* MG1363 (pAM401-*mob*) transconjugant

6. *S. faecalis* (pAM401-*mob*) transconjugant

7. *S. faecalis* recipient

8. *S. lactis* MG1363 recipient

9. *S. lactis* MG3020 recipient

10. pAM401 DNA (control)

11. *S. lactis* MG1363 chromosomal DNA (control)

1. *E.coli* S17-1 (pAM401-*mob*) transformant

2. *E.coli* S17-1 (pAM401-*mob*) transformant

3. *E.coli* S17-1 (pAM401-*mob*) transformant

4. *E.coli* S17-1 (pAM401-*mob*) transformant

5. *E.coli* S17-1 (pAM401-*mob*) transformant

6. *E.coli* S17-1 (pAM401-*mob*) transformant

7. *E.coli* S17-1 (pAM401-*mob*) donor in mating to *S.lactis*

8. *E.coli* S17-1 (plasmid free)
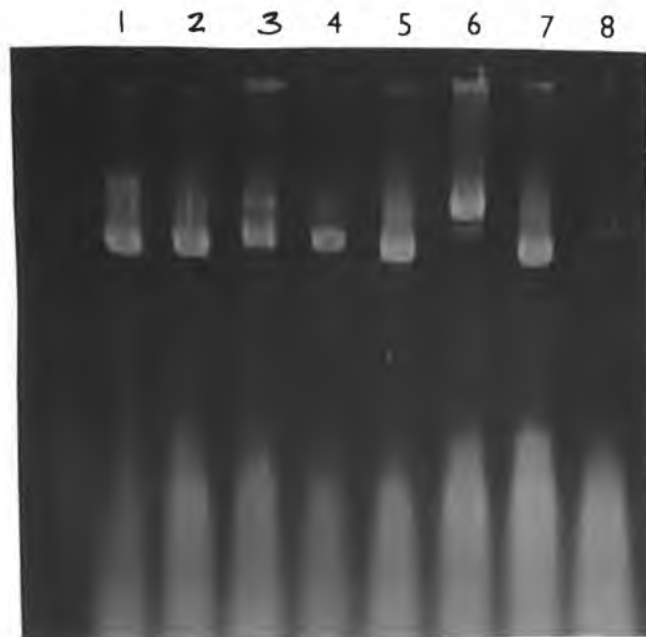
1. *E.coli* S17-1 (pAM401-*mob*) transformant

2. *E.coli* S17-1 (pAM401-*mob*) transformant

3. *E.coli* S17-1 (pAM401-*mob*) transformant

4. *E.coli* S17-1 (pAM401-*mob*) transformant

5. *E.coli* S17-1 (pAM401-*mob*) transformant

6. *E.coli* S17-1 (pAM401-*mob*) transformant

7. *E.coli* S17-1 (pAM401-*mob*) donor in mating to *S.lactis*
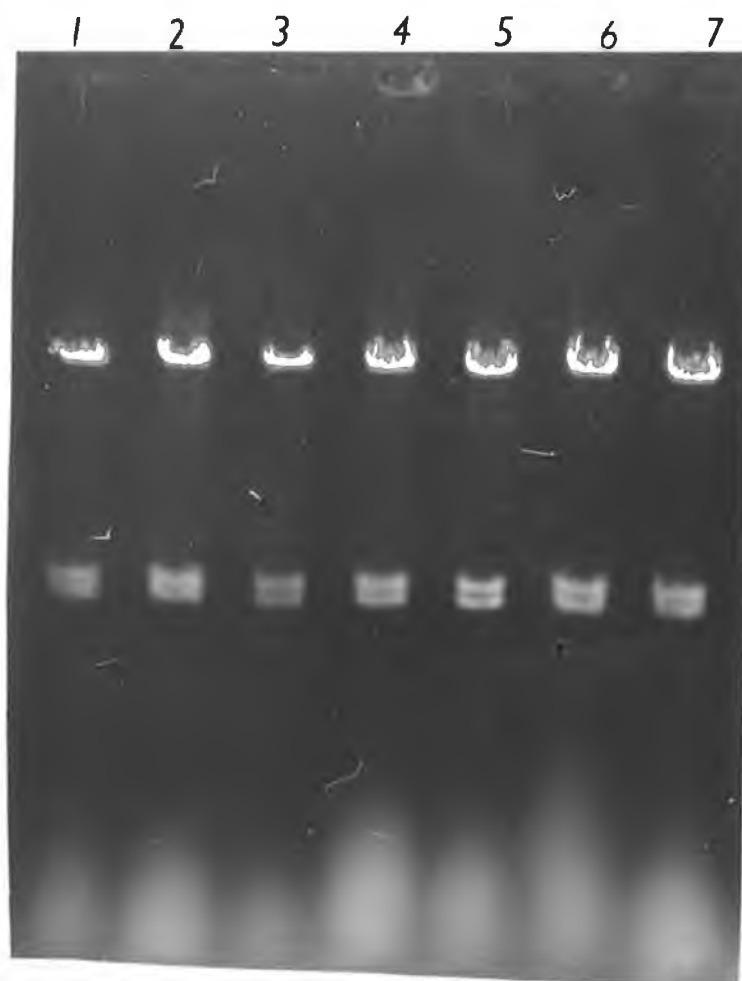
R. 8. EFFECT OF ORIENTATION OF Mob IN pAM401-mob ON TRANSFER FREQUENCY

In such a small fragment (<1kb) it might be difficult to determine orientation based on restriction analysis of suitable restriction sites within the mob region. Furthermore, a restriction map of the mob region was not available. Therefore, to analyse the effect the following strategy was adopted. pAM401-mob DNA was restricted twice (to ensure complete restriction) with *Bam*H1. The mixture was heat treated and vortexed to separate sticky ends and then re-ligated. This and a similar restiction which wasn't re-lighted was used to transform S17-1. The latter mixture yielded no transformants. A random sample of 10 transformants from the ligation mixture were used in matings with JM83 to check for variations in transfer frequency. Frequencies ranged between 2×10^{-2} and 1×10^{-3}. It was assumed these strains represented mob in different orientations and that, therefore, orientation has no significant effect on transfer frequency.

R. 9. ATTEMPTS TO IMPROVE EFFICIENCY OF TRANSFER BETWEEN *E. coli* AND *S. lactis*:

Conjugation efficiency depends on many factors, some of which have been discussed in the Introduction. Various combinations of plasmids, donors and recipients undoubtedly have different optimal requirements for conjugation to occur. Efficiency will be related to the extent conditions conform to these

requirements. In this experiment the following parameters were tested independently for influence on the efficiency of transfer of pAM401-mob from S17-1 to *S. lactis* MG1363.

(a) Concentration of Selective Antibiotic (Cm):

By MIC estimation it was observed that 4 ug/ml of Cm was the lowest concentration which inhibited *S. lactis* growth. When concentration used in experiments was lowered from 10 ug/ml (used in initial experiments) to 4 ug/ml no variation in efficiency of transfer was observed.

(b) Culture Age:

Growth curves were determined for S17-1 (pAM401-mob) and *S. lactis* 1363. Transfer frequencies obtained when mating donor and recipient strains at various phases of growth, determined by optical density at 600mm, are recorded on Table 7. Variations are not significant.

(c) Cell Density and Cell Ratio:

To vary density and ratio of donor and recipient in the mating mix different volumes of mid-log phase cultures of donor and recipient were used. Table 8 shows results.

TABLE 7   Effect of Culture Age on Transfer Frequency

Growth Phase of S17-1 (pAM40-mob)	Growth Phase of *S. lactis* MG1363	Transfer Frequency [a]
Stationary	Stationary	1×10^{-7}
Mid-log	Stationary	7×10^{-8}
Stationary	Mid-log	2×10^{-7}
Mid-log	Mid-log	1×10^{-7}

(a)   Frequency expressed as transconjugant/recipient.

TABLE 8   Effect of Cell Density on Transfer Frequencies

Donor (S17-1 (pAM401-mob)) (mls)	Recipient (MG1363) (mls)	Transfer Frequency [a]
0.1	0.1	6×10^{-8}
0.1	1.0	$<10^{-8}$
0.7	0.7	6×10^{-8}
1.0	0.1	2×10^{-7}
1.0	1.0	5×10^{-8}

(a) Transfer frequency expressed as transconjugants/recipient and

     is a mean of three independent matings.

(d) Transfer of pAM401-mob from S17-1 to MG1363 Protoplasts:

Because conjugation appears to be a non-specific event it is unlikely that a specific receptor exists on the recipient cell with importance to conjugation. A possible barrier to conjugation between gram-nagative and gram-positive bacteria is the difference in cell wall structure. (Although the divisions between gram-negative and gram-positive has a significance far beyond the cell wall chemistry, e.g. gram-positive bacteria have no outer membrane). The major part of the wall is made up of two covalently linked components, peptidoglycan and teichoic acid. Peptidoglycan forms at least 50% of the wall mass. It's cross linked structure provides a tough fibrus fabric giving strength and shape and several layers cover the cell. Attached to the peptidogylcan is an acidic polymer, often 30-40% of cell wall mass, usually teichoic acid.

The gram-negative envelope is far more complex; an outer and inner (cytoplasmic) membrane sandwich a peptidoglycan layer which accounts for about 5% of cell wall mass. The outer membrane is composed of a lipid bilayer with hydrophilic groups on the outer surface.

In a conjugation between gram-negative and gram-positive bacteria a likely barrier would be the much thicker peptidoglycan layer of gram positives. The outer surfaces in both divisions are also very different. A possible approach to improving the efficiency of transfer between these groups would be to remove the gram-positive peptidoglycan layer and expose the gram-positive membrane, which in many respects is similar to the gram negative outer membrane. Gasson (1980) has observed that transfer of pAMβ1 occurred between protoplasts of *S. lactis* at a high efficiency and without the requirement of a filter mating

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technique. Protoplasts have had the peptidoglycan layers removed by lysozyme and only the cytoplasmic membrane surrounds the cell.

Protoplasts of *S. lactis* MG1363 were generated and used in mating experiments with intact *E. coli* S17-1 (pAM401-mob) as described in Methods (M. 11). An initial experiment yielded a transfer frequency of 3×10^{-4} tansconjugants per recipient based on phenotypic evidence. However, at least four repetitions of this experiment failed to yield significant variations in the transfer frequency as compared to non-protoplasted recipients.

R. 10.   EXAMINATION OF HOST-RANGE OF pAM401-mob:

It was attempted to transfer pAM401-mob to several species of gram-positive bacteria in order to determine it's host range. Plasmid pAM401-mob contains the origin of replication of the broad host range gram-positive conjugative plasmid pIP501 so replication of pIP501 in all strains tested should not be a limitation. Conditions for matings are summarized in Table 9 for all recipients tested. *E. coli* S17-1 (pAM401-mob) was used as donor.

Under the conditions tested transfer was recorded to *S. lactis* MG1363 (10^{-7} per recipient), *S. lactis* MG3020 (10^{-7}) and *S. faecalis* (5×10^{-8}).

DNA was prepared from transconjugant and is shown on Plate 7. Transfer was not recorded to any other strain under these conditions.

Table 9: Conditions for mating between *E.coli* S17-1 (pAM401-*mob*) and gram-positive recipients

Recipient strain	O/N culture	Mating medium*	Medium● for counter-selection against donor	Cm [μg/ml] ⊙ for selection
S. lactis MG1363	BHI	BHI	GM17, Nal [50 μg/ml]	5
S. lactis MG3020	BHI	BHI	GM17, Nal [50 μg/ml]	5
S. faecium NCIB10415	MRS	BHI	GM17, Nal [50 μg/ml]	10
S. faecalis NIHE	BHI	BHI	GM17, Rif [100 μg/ml]	5
L. plantarum ATCC8014	MRS	BHI,MRS	MRS, Nal [50 μg/ml]	10
L. caesei NCIB9713	MRS	BHI,MRS	MRS, Nal [50 μg/ml]	10
L. acidophilus NCIB8795	MRS	BHI,MRS	MRS, Nal [50 μg/ml]	10
B. subtilis 168	BHI	BHI	MMB, Methionine	5
B.subtilis SO113	BHI	BHI	MMB, Tryptophan	5

* Best media for support of both donor and recipient, two media were tested in some matings

● Media were shown to allow good growth of the recipient and poor growth of the donor

⊙ MIC estimations with respect to Cm were carried out for all strains

DISCUSSION

Genetic studies in gram-positive bacteria have been hampered by the lack of suitable gene transfer techniques. Direct cloning in gram-positive bacteria, for example in *B. subtilis*, is not very successful when compared to *E. coli*. The usual approach is to carry out cloning and DNA manipulation in *E. coli* and later, using a shuttle vector, transform the DNA back to *B. subtilis*. This approach has been possible with a limited range of gram-positive bacteria which can be transformed and for which suitable shuttle vectors are available. For a more generally applicable approach conjugation would seem to hold the greatest potential. Conjugation is possible to a wide range of gram-positive bacteria and several broad host range conjugative plasmids have been isolated (Hershifield, 1979). The adaptation of broad host range conjugative systems has proved very successful (Simon *et al*, 1983) in gram-negative bacteria in overcoming restrictions on gene transfer. A general strategy would be to carry out DNA manipulations in *E. coli*, load DNA of interest onto a suitable shuttle vector and transform an intermediate gram-positive host. From here transfer the vector by conjugation to the target host. Such a system has certain pre-requisites and may require improvements in available technology. For example, a suitable intermediate host would need to be easily transformable to be able to maintain it's plasmid DNA complement intact and have the potential to donate plasmid DNA by conjugation to the target host. A suitable shuttle vector would need the ability to replicate in both *E. coli* and gram-positive hosts and have selectable markers for all three hosts. It would also need to be mobilizable from the intermediate to the target host.

If such a strategy is to be successful the individual elements need to be examined separately and each step analysed for it's applicability to the overall system.

The exerimental results presented in this thesis originate from an attempt to address this question. Several transformation protocols for *Streptococcus lactis* have been reported, from Kondo & McKay (1982, a) to Sanders and Nicholson (1987). It is apparent from this literature that efficiencies are highly variable and reproducibility very poor, between experiments and in different laboratories. It was, therefore, necessary to examine this technique and adapt a protocol. The protocol used was supplied by C. Geoghegan (personal communication). A transformation frequency of 1×10^5 transformants per ug of DNA was recorded when this procedure was used in the laboratory where the procedure has been established. However, reflecting comments in the literature, it proved difficult to use this procedure at NIHE. The reason for this could be related to water quality and impurities in particular batches of chemicals used as similar problems have been experienced by other workers (Ger. Fitzgerald, personal communication). The correction of such problems could prove time consuming so it was decided to examine transformation of *B. subtilis* as an alternative to *S. lactis*.

Transformation of *B. subtilis* proved much more successful. Competent cells could be easily generated and stored for transformation. Three different plasmids, the shuttle vector pAM401, the naturally occurring plasmid pAMB1 and the derived vector pIL253 were successfully transformed to *B. subtilis* at good frequencies. However, to be a successful intermediate host for a gene delivery strategy. *B. subtilis* would need to maintain plasmid DNA intact. Plasmid analysis by agarose gel electrophoresis showed that pAM401 and pIL253

remained intact whereas pAMB1 seemed to have decreased in size. Subsequent hybridization studies showed that pAMB1 had indeed undergone deletions (these are discussed in more depth below).

Several broad host range conjugative plasmids have been isolated from group B, D, and H streptococci (Hershfield, 1979). There have not been reports of broad host range conjugative plasmids in other gram-positive bacteria to date. Physical and genetic analysis of two of the streptococcal plasmids have been carried out: plasmids pAMB1, isolated from *S. faecalis* DS-5 (Clewell *et al,* 1974) and pIP501, isolated from *S. agalactiae* (Lanefield, group B) (Horadniceanu *et al,* 1976). Plasmid pIP501 has been characterized by Macrina *et al* (1983). A restriction map of pAMB1 plus a map localizing the Em^R Determinant and origin of replication of pAMB1 were published by LeBlanc and Lee (1984). Studies of the mechanisms of transfer of these plasmids have not been reported. Plasmid pAMB1 was chosen for study in this work because physical and genetic data were available and it has been shown to transfer from *B. subtilis* and *S. lactis* to several gram-positive recipients (Oultram & Young, 1985) in filter mating experiments.

In this study conditions which allowed transfer of pAMB1 from *S. lactis* to several gram-positive recipients were established. Reports of transfer to these hosts in literature have employed a variety of protocols. The procedure used here was a modification and simplification of the method of Oultram and Young (1985) and was more generally applicable to several intergeneric matings. Except with *C. acetobutylicum,* only the method of Oultram and Young worked in this case. Frequencies of transfer were similar to those previously reported. Recipients were analysed for the presence of pAMB1 DNA using ^{32}p labelled pAMB1 in hybridization studies because it was difficult to verify the presence of

pAMB1 DNA using agarose gel electrophoretic analysis of "mini-preped" DNA. Hybridization studies revealed intact pAMB1 in *S. faecalis*, *S. lactis* and *Bacillus thuringensis*. However, analysis of *B. subtilis* transconjugants revealed deleted forms of pAMB1.

Deletions in pAMB1 have been recorded previously upon transformation of *S. sanguis* (Macrina *et al*, 1980) and conjugation and transformation to *B.subtilis* (Van der Leli & Venema, 1987). However, intact pAMB1 has also been shown in *B. subtilis* (Oultram *et al*, 1987). In these reports deletions have affected transfer abilities. If *B. subtilis* was to be used in analysis of pAMB1 transfer or as an effective intermediate host for mobilization experiments it was necessary to determine the behaviour of pAMB1 in this host, especially given that previous reports were inconsistent. In this study pAMB1 transformants and transconjugants were shown to receive specific deletions independent of the gene transfer technique. However, deletions were more extensive than those reported by Van der Leli and Venema (10.6 kb), resulting in the loss of approximately 17 kb of DNA. The total loss was the result of more than one deletion although it was impossible to map deletions based on the data available.

It is unlikely that restriction modification causes these deletions as Van der Lelie and Venema (1987) transformed a restriction negative strain of *B. subtilis* to give similar deletions as they recorded for conjugation. In *B. subtilis* and *S. sanguis* (Behuke, 1981) ccc DNA is nicked on the cell surface and transported into competent cells as a single strand. Such processing could be related to post-transformational plasmid alterations. It has been suggested that pAMB1 may also use a single stranded mechanism of conjugal transfer which is common to many conjugative plasmids (Orzech & Burke, 1984). Van der Lelie and Venema have shown that it is unlikely that deletion formation is transposon - mediated.

Hahn and Dubnau (1985) have shown that, in a cointegrate plasmid they tested, specific deletion formation was related to intramolecular *rec*-independent recombination in *B. subtilis*, occurring between sites which contain a short region of homology. It was suggested this was not the only factor. Specific host recombination enzymes may be involved which recognise short direct repeats on the same molecule and that it might be possible to isolate host mutants lacking these specialized recombination functions. Such mutants would be better suited to the maintenance of intact plasmids. Oultram (personal communication) has observed a deterioration with time in the ability of a pAMB1 - containing *B. subtilis* strain to donate in matings over time. He is of the opinion that the intact plasmid is the only one involved in transfer and that deletions in *B. subtilis* are picked up quite slowly. In light of results presented here it seems more likely that deletions are dependent on factors which have diverged in the *B. subtilis* 168 strain over continuous sub-culturing in different laboratories. Considering that deletions were specific for transformats and transconjugants in one laboratory, different specific deletions in another laboratory suggests a strain variation. Deletions reported in this study appear to have affected the transfer genes of pAMB1 as attempts to mate *B. subtilis* transconjugants with *S. lactis* or other *B. subtilis* strains was unsuccessful. When pAMB1 was introduced by conjugation to strains of *S. lactis*, *S. faecalis* and *B. thuriengiensis* no deletions were observed. One of these strains may provide a more useful background in which analysis of pAMB1 transfer mechanisms may be analysed and mobilization studies carried out. Alternatively it may be possible to isolate *B. subtilis* mutants which do not cause deletions in plasmid DNA.

The vast majority of the MLS resistance plasmids reported in the literature seem to transfer efficiently to streptococcal recipients with frequencies ranging from 10^{-2} to 10^{-5} per recipient (Engel *et al*, 1980). A striking property of many

MLS resistance plasmids, isolated from different serogroups and of different geographical origin, is their uniformity in size (16 to 22 Md) (Engel *et al*, 1980). pAMB1 shares at least 95% homology with a similar sized plasmid pAC1 isolated from a group A *streptococcus* (Yagi *et al*, 1975) and there is a partial identity of the restriction endonuclease map of pR1405 and pAMB1 (Engel *et al*, 1980). Engel *et al* cited a personal communication which claimed plasmids pIP501 and pIP612 from group B streptococci had homology with plasmids pIP613 and pAMB1 originating from group D streptococci. Weisblum *et al* (1979) showed that several MLS resistance plasmids share a small common segment of DNA (2 to 3 Mdal) presumably the MLS determinant. In this study it has been shown that pAMB1 has homology with the 10 kb BstE11 fragment (outside the Em^R region) of pIP501. It was also shown that pAM401, which contains pIP501 DNA, but not the Em^R region, demonstrates homology with pAMB1. These results show that pAMB1 and pIP501 share DNA homology outside their MLS resistance determinants. The replication and copy control regions of pIP501 are also outside the 10 kb BstE11 fragment. It is suggested that homology is based on the transfer determinants which are likely to be conserved between similar classes of conjugative plasmids. Loss of the 20 kb region of pIP501 (including the 10 kb BstE11 fragment) removes transfer ability from pIP501. A deletion derivative of pIP501, pGB301, carries this deletion and was shown to be non-conjugative (Behnke, 1981). Hershfield (1979) has shown that pAMB1 and pIP501, plus several other MLS plasmids isolated from groups B, H and D streptococci, share bands of common sizes when restricted with *Hind*111 or *Hinc*11. All these plasmid were in the 17 Mdal size range and were transmissible to a broad host range.

Considering the problem of deletion formation in *B. subtilis* of pAMB1 it may be worth screening some alternative conjugative plasmids for increased stability.

The majority of these plasmids would appear to have certain areas conserved among them suggesting common ansceestory and probably transfer by similar mechanisms.

The recent finding that the mobilization functions of plasmid RSF1010 of incompatibility group IncQ can mediate transfer of plasmids from *Agrobacterium* to plants indicates that conjugation is a non-specific process (Buckanan-Wollaston *et al*, 1987). It was, therefore, decided to examine the possibility of direct transfer by conjugation of plasmid DNA from *E. coli* to gram-positive bacteria. A vector was constructed which could replicate in both gram-positive bacteria and *E. coli*, had selectable antibiotic resistance markers for both and also contained the *mob* region of plasmid RP4. Transfer by conjugation of this plasmid, dependent on the RP4 transfer functions, was demonstrated to other *E. coli* strains and to the gram-positive bacterium, *S. lactis*. Transfer efficiency to *S. lactis* was low, in the order of 10^{-7} per recipient. Such a low frequency would not be useful for some of the envisaged applications of the system, for example, *in vivo* transposon mutagenesis. Attempts were therefore made to improve the frequency of transfer.

Several parameters were examined, including the orientation of the *mob* fragment, concentration of selective antibiotics, culture age, cell densities and cell ratios, and the employment of protoplasts of the *S. lactis* recipient. Variation of the former parameters failed to yield conditions which enhanced frequencies above those already recorded. The latter experiment, the employment of protoplasted recipients was aimed at removing cell wall barriers to conjugation. Gasson (1980) had previously observed increased frequency of transfer of pAMB1 between *S. lactis* protoplasts. Because transfer in this case appeared to be a non-specific event it seems unlikely that specific receptors were involved in

transfer and that close association of donor and recipient cell membranes might enhance transfer. Although an initial experiment yielded an increased transfer frequency it proved impossible to repeat this result. It may be that conditions for such a transfer were not being met in subsequent experiments although the same protocol was used. The main difficulty in this technique was the handling of the protoplasts and it is possible that the difficulties of reproducibility experienced with *S. lactis* protoplast transformatins may be experienced in protoplast conjugation systems also.

An examination of the host range to which transfer was possible demonstrated transfer to *S. lactis* and *S. faecalis*. Other gram-positive recipients tested, including species of *Lactobacillus* and *B. subtilis* failed to yield transconjugants. Simultaneous to this work Trieu-Cuot *et al* (1987) reported construction of a similar vector and it's transfer, by co-mobilization with the IncP plasmid pRK212.1, from *E. coli* donors to *S. lactis*, *Enterococcus faecalis*, *S. agalactiae*, *B. thuringensis* and *S. aureus*. Although this work extends the host range recorded in this study, the fact that several species tested did not yield transconjugants under conditions tested and the low transfer frequency recorded here and by Trieu-Cuot *et al* (10^{-7} per recipient) suggest such a system will lact versatility and have limited application. Nevertheless, where it can be applied this system would be undoubtedly easier to handle than protoplast transformation. Furthermore, all the shuttle vectors described so far, which are adapted to a variety of strategies and particular problems, can theoretically be used similarly to pAM401-mob, provided that a functional *mob* region is inserted. Trieu-Cuot *et al* used a co-resident plasmid in *E. coli* to induce mobilization. In this study it was possible to use *trans* acting transfer functions which had been integrated into the *E. coli* chromosome.

Trieu-Cuot *et al* make no reference to the mechanism of transfer which may be involved. It has been reported recently (Gruss & Ehrlick, 1988) that with *B. subtilis* plasmids, which replicate via a single strand intermediate, there is a shift in plasmid distribution from principally monomeric (for wild type) to principally multimeric (for hybrids) forms upon insertion of *E. coli* DNA fragments. It was proposed that multimers were a product of impaired termination of rolling-circle replication. Results obtained in this study show that *S. lactis* transconjugants which have received pAM401-mob appear to have a much greater proportion of high molecular weight multimers than, for example, have *S. lactis* strains which have been transformed with pAM401. Furthermore, upon transformation of *E. coli* with pAM401-mob DNA isolated from *S. lactis* transconjugants, one *E. coli* transformant isolated contained only the multimeric form of pAM401-mob. These observations may reflect the mode of transfer of pAM401-mob, suggesting that it is transferred as a single-stranded intermediate from *E. coli* and replicates by the rolling circle model in *S. lactis*. This would imply that the mechanism of transfer of pAM401-mob from *E. coli* to *S. lactis* is similar to the mechanism of conjugative transfer of plasmid DNA between gram-negative bacteria.

CONCLUSIONS

The major aspects of gene transfer among gram-positive bacteria have been examined. Transformation experiments demonstrated the problems with reproducibility when using protoplast transformation of *S. lactis*. Transformation of *B. subtilis* competent cells was more successful. The use of conjugation as a means of transferring plasmid DNA between gram-positive bacteria was examined using the broad host range conjugative plasmid pAMB1. Problems were experienced when pAMB1 was transferred to *B. subtilis* by transformation or conjugation in that major specific deletions were incurred in the plasmid. This compared with similar observations by other workers, although a separate report had reported the maintenance of intact pAMB1 in *B. subtilis*. These results suggested that the use of *B. subtilis* as an intermediate host in a system for transfer of plasmid vectors from *E. coli* to gram-positive bacteria might not be a good choice. Although *S. lactis* maintained intact pAMB1 and was a good donor in matings poor reproducibility in transformation experiments excluded it from a similar system. It may be feasable to isolate *B. subtilis* strains with increased ability to maintain intact plasmid DNA or it may be possible to choose conjugative plasmids which are more stable in *B. subtilis*. However, for the moment it appears that the technology available for the transfer of plasmid vectors from *E. coli* to gram-positives, employing *B. subtilis* or *S. lactis* as an intermediate host is not dependable.

As an alternative strategy it was attempted to transfer a plasmid vector directly from *E. coli* to a range of gram-positive bacteria by conjugative mobilization. This proved successful in the case of *S. lactis* using the RP4 *mob* region cloned in the vector which could be mobilized by *trans* acting transfer functions

integrated in the *E. coli* chromosome. This approach circumvents some of the problems associated with using an intermediate host but low transfer frequencies and limitations on host range may decrease the versatility of this type of system. Preliminary observations suggest the mechanism of conjugation is similar to conjugation in gram-negative bacteria.

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APPENDIX I

ANTIBIOTIC ABBREVIATIONS AND CONCENTRATION OF STOCK SOLUTIONS

Antibiotic (abbreviation)		Concentration of stock (mg/ml)
Chloramphenicol	(Cm)	50
Erythromycin	(Em)	30
Streptomycin sulphate	(Sm)	600
Spectinomycin	(Sp)	600
Tetracycline	(Tc)	20
Rifampacin	(Rif)	400
Nalidixic Acid	(Nal)	50

APPENDIX II

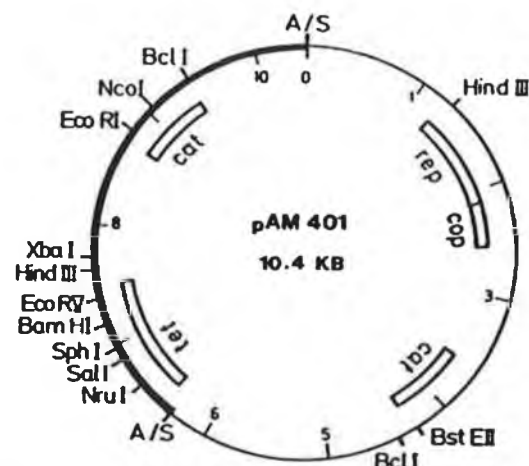Restriction map of pAMβ1-reproduced from LeBlanc and Lee, 1984.
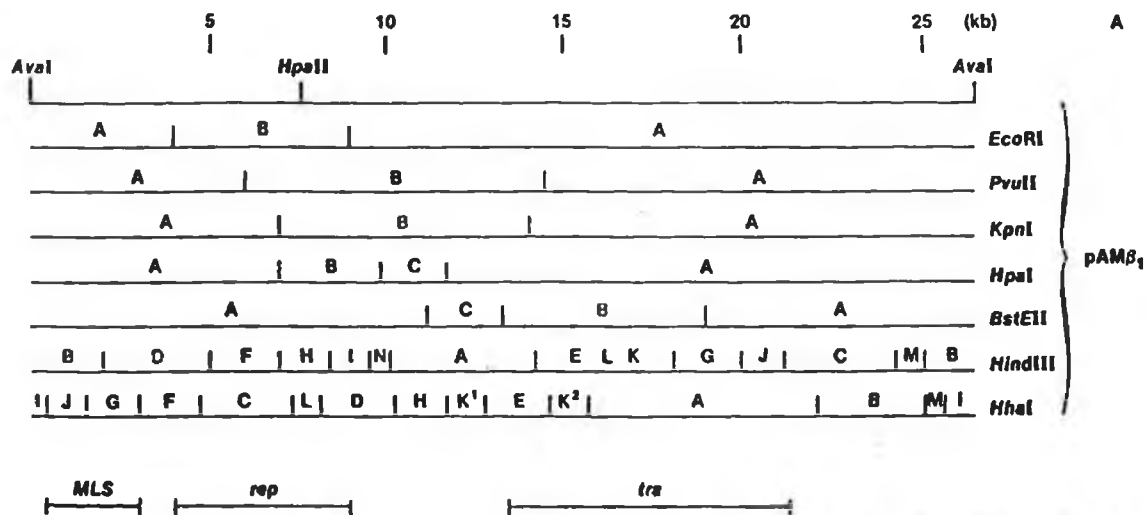
Gram-positive cloning vectors-reproduced from Harlander, 1986.
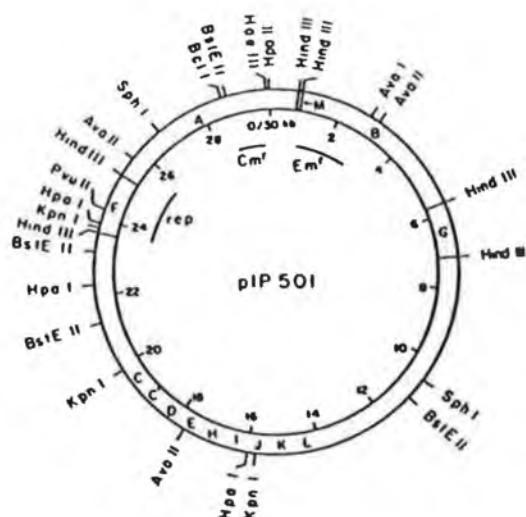
Vector: pIP501
Size: 30 kb
Single sites for gene inactivation: Cm^r: *Bst*EII
Other single sites: *Ava*I, *Bcl*I, *Hae*III, *Hpa*II, *Pvu*II
Reference: R. P. Evans and F. L. Macrina, J. Bacteriol. 154:1347–1355, 1983

Vector: pAM401
Size: 10.4 kb
Single sites for gene inactivation: Cm^r (pGB354): *Bst*EII; Cm^r (pACYC184): *Eco*RI, *Nco*I; Tc^r: *Bam*HI, *Eco*RV, *Sal*I, *Sph*I
Other hosts: *E. coli*
Reference: R. Wirth, F. Y. An, and D. B. Clewell, J. Bacteriol. 165:831–836, 1986
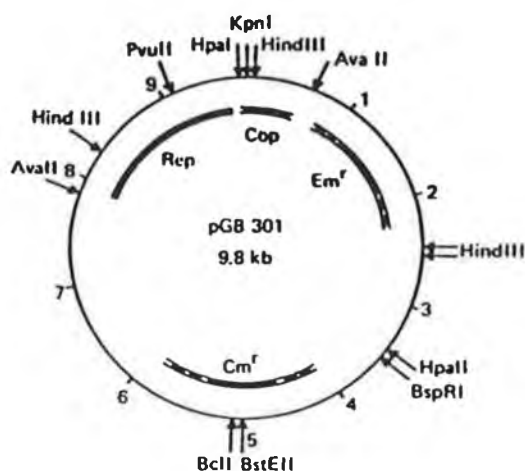
Vector: pGB301
Size: 9.8 kb
Single sites for gene inactivation: Cm^r: *Bst*EII
Other single sites: *Bcl*I, *Bsp*RI, *Hpa*II, *Ava*I
Other hosts: *Staphylococcus* spp., *Bacillus* spp.
Reference: D. Behnke, M. S. Gilmore, and J. J. Ferretti, Mol. Gen. Genet. 182:414–421, 1981
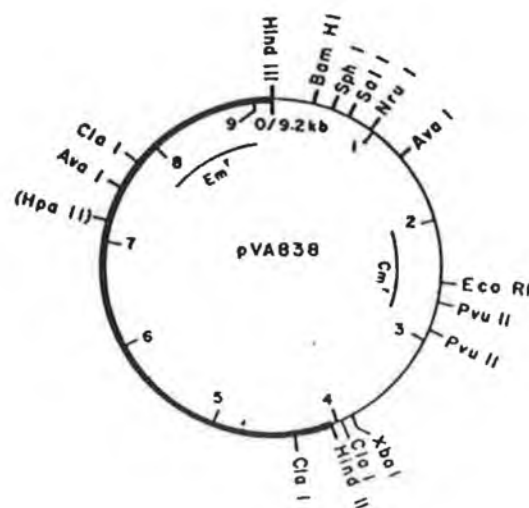
Vector: pVA838
Size: 9.2 kb
Single sites for gene inactivation: Cm^r: *Eco*RI, *Pvu*II
Other single sites: *Bam*HI, *Hpa*II, *Nru*I, *Sal*I, *Sph*I, *Xba*I
Other hosts: *E. coli*
Reference: F. L. Macrina, J. A. Tobian, K. R. Jones, R. P. Evans, and D. B. Clewell, Gene 19:345–353, 1982