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The construction and properties of an integrable plasmid for genomic analysis in Staphylococcus aureus

John Bernard Luchansky

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THE CONSTRUCTION AND PROPERTIES OF AN INTEGRABLE PLASMID
FOR GENOMIC ANALYSIS IN STAPHYLOCOCCUS AUREUS

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The construction and properties of an integrable plasmid for genomic analysis in Staphylococcus aureus

by

John Bernard Luchansky

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

The integrable plasmids originally developed for genetic analysis of *Bacillus subtilis* have the following properties: 1) they are selectively maintained in *Escherichia coli* because they contain both a Gram-negative origin of replication and a selectable marker(s); 2) they can also be selected in *B. subtilis* because they contain a Gram-positive selectable marker(s); 3) they cannot be maintained autonomously in *B. subtilis* because they do not harbor a Gram-positive origin of replication; and 4) they can be inherited in *B. subtilis* because they contain a fragment of DNA homologous to the *B. subtilis* chromosome (Saunders et al., 1984b). Insertion vectors are established in a Gram-positive host upon recombination between DNA sequences present on the plasmid and homologous chromosomal sequences. Integrable plasmids have found use for cloning promoters and genes involved in sporulation (Youngman et al., 1985b), generating random transcriptional fusions (O’Kane et al., 1986), cloning origins of replication (Niaudet and Ehrlich, 1979), stabilizing the inheritance of heterologous genes (Saunders et al., 1984a), chromosomal mapping (Haldenwang et al., 1980), and generating deletions (Stahl and Ferrari, 1984).

It has been difficult to apply recombinant DNA methodology toward genetic analysis of *Staphylococcus aureus* NCTC 8325 because the genetics and molecular biology of this organism are not well-characterized, especially when compared to *E. coli* or even *B. subtilis*. Progress has also been hampered by the poorly defined and low-frequency transformation system of *S. aureus*. The specific aim of this study, therefore, was to develop an
integrable plasmid to facilitate genomic analysis of *S. aureus*. A novel, temperature-sensitive integrable plasmid (designated pPQ126) was constructed by removing a 1.3 kilobase-pair (kb) fragment from plasmid pTVlts and replacing it with a 2.5 kb gentamicin-resistance determinant from transposon Tn4001. The recovery of the recombinant molecule pPQ126, directly in *S. aureus*, was accomplished by using a restrictionless recipient strain and an improved procedure for protoplast transformation that was developed in this study. Plasmid pPQ126 is recovered as an autonomous replicon following transduction into an appropriate recipient strain at the permissive temperature (30°C). When cells containing autonomous pPQ126 are shifted to non-permissive conditions (39°C), portions of Tn917 or Tn4001 direct the integration of pPQ126 into homologous target sequences (chromosomal insertions of Tn551 or Tn4001). By insisting for retention of pPQ126 at 39°C on media containing gentamicin, erythromycin, and chloramphenicol, it is possible to recover cells that contain pPQ126 integrated at a predetermined chromosomal site. This study describes the construction, transfer, and properties of pPQ126 in *S. aureus*. 
LITERATURE REVIEW

*Staphylococcus aureus* is a Gram-positive, non-motile, catalase-positive, facultatively anaerobic coccus usually arranged in irregular clusters. *S. aureus* is a potent human and animal pathogen responsible for infections ranging from boils (Noble and White, 1983) and food poisoning (Iandolo and Dyer, 1981) to toxic shock syndrome (Gemmell, 1982; Todd et al., 1978) and endocarditis (Karchmer, 1985). Although one of the earliest recognized and best studied human pathogens, *S. aureus* remains a common agent of severe infection in immunocompetent patients (Cohen, 1986; Kaplan and Tenenbaum, 1982). *S. aureus* also elaborates a variety of interesting macromolecules (Arvidson, 1983; Gemmell, 1985), including protein A (Langone, 1982), lipases (Kotting et al., 1985), coagulase (Jeljaszewicz et al., 1983), and several hemolysins and enterotoxins (Bergdoll, 1985; Bergdoll et al., 1985; Rogolsky, 1979; Rogolsky, 1985; Todd, 1985).

Because of the clinical significance as well as the commercial applications of this organism, considerable effort has been directed to analyze the genome of *S. aureus*. Molecular cloning as an approach to genomic analysis in *S. aureus*, however, has been largely unexplored. A practicable cloning system developed for *S. aureus* would facilitate the study of the molecular biology and genetic organization of this complex and interesting microorganism.

### Cloning Vehicles

Several obstacles must be circumvented to successfully clone a particular fragment of DNA: initially, the DNA must be introduced into the
interior of host cells, and then once inside the cell, this DNA must be both expressed and maintained to be detected and studied. Under most circumstances, the cloning vector imparts the replicative functions to the recombinant molecule. Because the nature of the cloning vehicle will place constraints on both the size and analysis of the DNA fragment to be cloned, the selection of a suitable cloning vector is of paramount importance. Bacteriophage (e.g., charon phage), plasmids (e.g., pBR322), and bacteriophage-plasmid combinations (e.g., cosmids and phasmids) have served as suitable cloning vehicles for the in vitro manipulation and study of DNA fragments. This discussion will be limited to specialized plasmid vectors (integrable plasmids); the other vectors listed above will be considered only briefly.

Charon phage

Charon vectors are derivatives of wild-type phage lambda that have been specifically altered for cloning purposes. Recombinant molecules are constructed by either replacing a segment of nonessential lambda DNA (defined by restriction sites) with a similar length of passenger DNA (replacement vectors), or by the insertion of passenger DNA into a single restriction site of a deleted derivative of lambda (insertional vectors). Lambda derivatives have three main advantages over plasmid vectors for cloning purposes: 1) thousands of recombinant phage plaques on a single petri plate can easily be screened for a given DNA sequence by using hybridization probes; 2) in vitro packaging of recombinant DNA molecules provides a very efficient means of introducing cloned DNA into bacteria; and 3) millions of independently packaged recombinant phage can be
replicated and conveniently stored in a single solution as a library in which all sequences of a large genome are likely to be represented. Additional material on lambda packaging and general information on charon phage are available in reviews by Rosenberg et al. (1985) and Blattner et al. (1977), respectively.

**Cosmids**

Cosmids are specially constructed plasmids containing the phage lambda cos site, an origin of replication, and a selectable marker, such as an antibiotic-resistance determinant. The cos site is required for packaging recombinant molecules into phage lambda particles for the introduction of the recombinant DNA into an appropriate recipient strain. Thereafter, cosmids are replicated as typical plasmids. In this system the recombinant vector, a plasmid containing the lambda cos site plus passenger DNA, is packaged in vitro into lambda heads and then is transduced into a recipient cell. The advantage of the cosmid system over other lambda derivatives (e.g., charon vectors) is the smaller size of cosmids, allowing for larger segments of passenger DNA to be cloned. An advantage of the cosmid system over plasmid cloning vehicles is the high selectivity of the cosmid packaging system for recombinant molecules. This last advantage eliminates complicated screening procedures, and the pretreatment of vector (e.g., removal of 5'-phosphate groups with alkaline phosphatase) to prevent vector-vector ligation. For a more thorough discussion of cosmid packaging and cosmid cloning, the reader is referred to the following articles: Feiss et al., 1982; Murray, 1983; and Palva and Liljestrom, 1981.
Phasmids

Additional phage lambda/plasmid combinations that consist of a plasmid plus the lambda attachment site (att site) have been constructed. Such plasmids can insert into an intact lambda genome via the usual lambda mode of integration: the term phasmid refers to the moiety generated by the reversible recombinational insertion of the plasmid into the lambda genome. Advantages of the phasmid system are that the recombinant molecules can be propagated as phage or plasmids, and that these molecules can be conveniently stored almost indefinitely as phage particles. A disadvantage of the phasmid system is the occurrence of spontaneous rearrangements. The interested reader is referred to the following articles for more information concerning phasmids: Brenner et al., 1982; and Kahn and Helinski, 1978.

Plasmids

Plasmids are extrachromosomal, autonomously replicating, covalently closed circular (CCC), double-stranded DNA molecules, ranging in size from 2 to 200 kilobases (kb) in length. Although only smaller segments of DNA can be cloned with plasmids (approximately 15 kb) as compared with charon (approximately 25 kb) or cosmid (approximately 45 kb) vectors, plasmids are often easier to manipulate because a viral life cycle and packaging process are avoided (Maniatis et al., 1982). An efficient plasmid cloning vehicle would contain several features: 1) one or more readily selectable markers (such as antibiotic resistance determinants); 2) one or more unique sites for restriction endonucleases; 3) a relatively low molecular weight [less than 10 megadaltons (md)] to facilitate the isolation, transfer, and
analysis of the recombinant molecule, as well as the accommodation of larger segments of passenger DNA; and 4) a relaxed mode of replication so the plasmid replicates in high copy number for the isolation of large amounts of plasmid DNA, and to facilitate the purification of proteins encoded by cloned genes. In addition, the vector should be constructed such that only vectors containing cloned DNA confer a detectable phenotype upon the cloning strain (e.g., insertional inactivation vectors). Many cloning vehicles meet the first four requirements, but not all vectors fulfill the last requirement. It should be noted, however, that plasmid cloning vehicles have been constructed that replicate in low copy number (Hasnain and Thomas, 1986; Stoker et al., 1982). Low copy number vectors have been used for cloning genes encoding for products that would be deleterious if overproduced, such as surface structural proteins (e.g., ompA; Hashimoto-Gotoh et al., 1981), or the rat insulin gene (Brosius, 1984). Although low copy number plasmid vectors prevent cell death, decreased expression of the gene complicates the purification of gene products. This problem may be circumvented by using runaway plasmid vectors (Masui et al., 1983; Remaut et al., 1983; Uhlin et al., 1979). The control of plasmid copy number for runaway vehicles is lost at elevated temperatures, resulting in significant accumulation of plasmid DNA and plasmid gene products. Cell proliferation eventually ceases, but by this time plasmid DNA may account for up to 75% of the DNA in the cell (Uhlin et al., 1983).

Initially, recombinant plasmids were constructed by the controlled shearing of resistance factor (R-factor) DNA. Upon transformation into
competent cells of *Escherichia coli*, the sheared R-factor DNA recircularized, forming new autonomously replicating plasmids (Cohen and Chang, 1973). For example, the plasmid pSC101, encoding for resistance to tetracycline, was constructed by shearing plasmid R6-5 and subsequent *in vivo* recircularization upon transformation into *E. coli* (Cohen and Chang, 1973). With the discovery of restriction endonucleases, a procedure was described for the construction of "hybrid" or "chimeric" plasmids via *in vitro* annealing of DNA fragments from separate plasmids (Cohen et al., 1973). It was also determined that plasmids linearized with EcoRI would transform *E. coli*, but at a frequency 10-fold lower than CCC or nicked circular molecules of the same plasmid. The transformation frequency could be increased slightly if the restricted plasmid mixture was treated with DNA ligase before transformation (Cohen et al., 1973). From restriction endonuclease analysis, pSC101 was found to contain a single EcoRI site that was not within the genes encoding for plasmid replication or tetracycline resistance. Although it was one of the first plasmid cloning vehicles (Cohen et al., 1973), pSC101 has found little use for *in vitro* cloning because it carries only a single selectable marker, and insertional inactivation cannot be used to screen for clones carrying recombinant molecules.

Plasmid pBR322 is perhaps the best-characterized recombinant plasmid: pBR322 exhibits all of the salient features described earlier for an efficient cloning vehicle (Bolivar et al., 1977). pBR322 has facilitated the cloning, isolation, and analysis in *E. coli* of many genes from eukaryotes, prokaryotes, and viruses (Itakura et al., 1977; Kreiswirth et
al., 1983; Peden et al., 1980; Yelverton et al., 1983). More information concerning plasmids can be found in Helinski et al. (1985).

Recombinant DNA or genetic engineering methodology, as well as the depth of knowledge about the genetic systems involved are less sophisticated for most Gram-positive bacteria than for Gram-negative bacteria. Furthermore, genetic exchange mechanisms (e.g., transformation of plasmids) are more straightforward in E. coli than in Gram-positives; direct cloning into competent cells of Bacillus subtilis requires plasmid multimers (Canosi et al., 1978; Contente and Dubnau, 1979). For this reason, many Gram-positive cloning schemes utilize E. coli as the host organism for expression and analysis of cloned DNA fragments. Although many heterologous genes (DNA not originating from E. coli) are expressed in E. coli, it is often difficult to isolate the products of these cloned genes because generally they become trapped in the periplasmic space. Therefore, it would be of both practical importance and general interest to construct more sophisticated plasmid cloning vehicles (and more efficient genetic exchange processes) for the transfer and the subsequent analysis of recombinant vectors in a Gram-positive organism. To accomplish these goals, several recombinant plasmids have been constructed which serve as shuttle vectors between Gram-positive and Gram-negative bacteria (Ehrlich et al., 1976; Ishiwa and Shibahara, 1985; Macrina et al., 1982; Macrina et al., 1983; Wirth et al., 1986), between different Gram-positive bacteria (Harris-Warrick and Lederberg, 1978; Kreft et al., 1982; Łuczak et al., 1985; Vasseghi and Claverys, 1983), and between different Gram-negative bacteria (Danner and Pifer, 1982). Shuttle plasmids are hybrid vectors
that are replicated and selectively maintained in either of two different host strains because they encode for both a selectable marker(s) and origin(s) of replication functional in each host. Cloning Gram-positive genes into *E. coli* by using shuttle vectors obviates the requirement for plasmid multimers for successful transformation of *B. subtilis*. Recently, *E. coli-B. subtilis* shuttle vectors were constructed that function as runaway plasmids in *E. coli*; these versatile vectors should become invaluable for genetic engineering of both Gram-positive and Gram-negative organisms (Andreoli, 1985). Shuttle vectors have been useful for cloning a variety of interesting genes (Fouet et al., 1982; Kiss and Baldauf, 1983; Kreft et al., 1983). As discussed below, other hybrid vectors have been constructed that replicate in only one host (integration vectors).

**Integrable Plasmids**

Plasmid integration vectors (integrable plasmids or plasmid insertion vectors) are hybrid vectors that are selectively maintained and autonomously replicated in one host (replicating host), and that contain genetic determinants expressible in a different host (non-replicating host) under certain circumstances. Typically, the replicating host is a genetically well-defined Gram-negative organism (e.g., *E. coli*), and the non-replicating host is a Gram-positive organism (e.g., *B. subtilis*). Integrable plasmids result in the insertion of non-homologous DNA (e.g., Gram-negative origin of replication) into the chromosome of the non-replicative host by recombination between the vector containing cloned DNA fragments (derived from the non-replicative host) and homologous chromosomal sequences of the non-replicative host.
One of the first reported integrable plasmids (p1949) was constructed for use in *B. subtilis* (Haldenwang et al., 1980). Ferrari et al. (1983) utilized an integrable plasmid, pJH101, to clone and map several genes in *B. subtilis*. A similar plasmid, pHV32, was used for insertional mutagenesis in *B. subtilis* (Niaudet et al., 1982); both pHV32 and pJH101 yielded equivalent results for the generation of auxotrophic mutations (Ferrari et al., 1983). Additional integrable plasmids have been utilized for molecular cloning and genomic analysis in Gram-positive systems (Fahnestock et al., 1986; Gryczan and Dubnau, 1982; LaFauci et al., 1986; Niaudet et al., 1982; Pozzi and Guild, 1985; Prozorov et al., 1983; Vasseghi and Claverys, 1983; Young, 1983). Integrable plasmids have found extensive use because they are genetically and physically well-characterized, and because they replicate in *E. coli*. Plasmid construction and purification can be accomplished more readily in *E. coli* than in any Gram-positive system. Insertion vectors have been used to clone the replication origins of other replicons, to determine the map position of cloned fragments of unknown origin, to generate deletions, to clone specific DNA sequences from different strains of the same organism, to stabilize the inheritance of genes that are not easily established on plasmid vectors, and to expand the chromosomal map of genetically ill-defined organisms (Haldenwang et al., 1980; Mejean et al., 1981; Niaudet and Ehrlich, 1979; Niaudet et al., 1982; Saunders et al., 1984a; Stahl and Ferrari, 1984).

The insertion plasmid pJH101 (see Figure 1) has several features that are desirable for both mapping and cloning studies (Ferrari et al., 1983).
Figure 1. Restriction map of pJH101

pJH101 is a 5,391 base-pair integrable plasmid encoding for resistance to ampicillin (AMP), tetracycline (TET), and chloramphenicol (CAT).

Approximately 4.3 kb of pJH101 (the AMP and TET genes and the origin of replication (Ori)) are derived from plasmid pBR322. The remaining 1.0 kb of pJH101 harbors the chloramphenicol acetyl transferase gene (CAT) from *S. aureus* plasmid pC194. Arrows demonstrate the direction in which genes are transcribed (taken from Ferrari et al., 1983).
pJH101 will be discussed in further detail because it exemplifies many of the attributes common to all insertion vectors. Approximately 4.3 kb of pJH101 are derived from pBR322; the remaining 1 kb consists of a chloramphenicol acetyl transferase gene (from the S. aureus plasmid pC194) cloned into the PvuII site of pBR322. pJH101 has three antibiotic-resistance markers (Ampicillin, Ap^R; Tetracycline, Tc^R; and Chloramphenicol, Cm^R), as well as several unique cut sites for restriction endonucleases, including a PstI site within the region coding for Ap^R, and at least three restriction sites within the tetracycline gene (HindIII, BamHI, and SalI). The restriction sites within the tetracycline determinant of pJH101 are best suited for the insertion of passenger DNA into this vector: 1) inactivation of the tetracycline determinant by the insertion of passenger DNA facilitates the rapid and facile screening of a large number of transformants containing putative recombinant plasmids; and 2) evidence suggests that overproduction of the tetracycline resistance gene product is lethal for B. subtilis (Ferrari et al., 1983; Kreft et al., 1983).

One approach for utilizing this vector consists of digesting both pJH101 and B. subtilis chromosomal DNA with a restriction endonuclease, and then ligating and transforming this mixture into E. coli with selection for Ap^R. Plasmid DNA is isolated from clones containing pJH101 plus passenger fragments (recombinant pJH101), and transformed into an appropriate recipient strain of B. subtilis with selection made for Cm^R. pJH101 does not contain an origin of replication for B. subtilis, thus precluding autonomous replication of recombinant pJH101 in this host.
Chloramphenicol-resistant transformants of *B. subtilis* are (most likely) the result of integration of recombinant pJHl01 into the host chromosome because areas of homology exist between the cloned fragment of passenger DNA (chromosomal DNA from *B. subtilis*) and the host chromosome. Integration is believed to occur via a Campbell-type (single crossover) recombinational event (Duncan et al., 1978; Ferrari et al., 1983; Haldenwang et al., 1980; Harris-Warrick and Lederberg, 1978), much like the integration of phage lambda. It should be noted, however, that passenger DNA alone can also integrate into the host chromosome via a double-crossover event often resulting in the exclusion of the non-homologous vector-only portion of the plasmid, and accompanied by the deletion of chromosomal material (Pozzi and Guild, 1985; Young, 1983). Pozzi and Guild (1985) constructed a donor-recipient system to test the mode of integration of plasmid DNA into the chromosome of *Streptococcus pneumoniae*: the double-crossover mechanism produced 600-fold more transformants than the single-crossover event. For the integration of pJHl01 derivatives into the chromosome of *B. subtilis*, a 2-to 10-fold decrease in recovery of transformants was observed when selection was made for the single-crossover event (Ferrari et al., 1983). If integration occurs via a Campbell-type recombination event, the entire plasmid, plus adjacent chromosomal DNA, can be recovered as follows: *B. subtilis* chromosomal DNA from a chloramphenicol-resistant transformant is digested with a restriction enzyme that does not cut within the plasmid, and after religation, the mixture is transformed into *E. coli* with selection made for resistance to ampicillin. Figure 2 depicts a Campbell-type recombination of a typical
Figure 2. Recombination and recovery of an integrable plasmid

The diagram shows a typical integrable plasmid that contains a Gram-negative selectable marker (ampicillin resistance; Ap$^{\text{R}}$), a Gram-positive selectable marker (chloramphenicol resistance; Cm$^{\text{R}}$), and a fragment of cloned DNA [denoted by arrows and bordered by BamHI (Bam) sites]. Jagged lines denote recognition sequences present on the chromosome for the restriction endonuclease BglII. Integration is directed into the chromosome via Campbell-type recombination between the cloned DNA on the plasmid and homologous chromosomal sequences. The entire plasmid and chromosomal DNA flanking the insertion site are recovered by digesting the chromosomal DNA with BglII (no BglII sites within plasmid sequences), ligating the fragments, and transforming the resulting mixture into E. coli (taken from Old and Primrose, 1986).
integrable plasmid into a homologous chromosomal target, and the removal
and subsequent recovery in *E. coli* of the vector plus adjacent chromosomal
DNA. The above approach provides a convenient and rapid means for cloning
*B. subtilis* chromosomal DNA into *E. coli* for further analysis. pJH101 was
successfully used in this manner to study the *spoOB* locus (Ferrari et al.,
1983), and several rRNA operons in *B. subtilis* (LaFauci et al., 1986).

**Transposable Elements**

Transposable elements have been identified in a variety of prokaryotic
and eukaryotic (yeast, maize, fruit flies) organisms (Calos and Miller,
1980; Kleckner, 1977; Kopecko, 1980). These elements do not exist
autonomously (e.g., apart from a plasmid or chromosomal replicon).
Kleckner (1981) grouped the prokaryotic transposable elements into four
major classes based on mechanistic differences, DNA sequence homologies,
and correlated relationships in genetic organization. Included in this
system are the insertion sequences (commonly referred to as IS elements,
Class I); the translocatable antibiotic resistance elements (commonly
referred to as Tn elements or transposons, Class II and some members of
Class I); a few bacteriophage such as phage Mu (Class III); and elements
that remain unclassified because of their uniqueness and/or the paucity of
information available for classification (Class IV). Transposons have been
defined as large DNA sequences that express a phenotypically identifiable
trait unrelated to their own insertion and that are capable of *recA-
-independent transposition, usually in either of two physical orientations,
as a discrete, linear non-permuted unit (Kopecko, 1980).
Figure 3 depicts the components of the structure of a typical transposon: a central region containing accessory genetic determinants, and in some instances information required for transposition; repeated terminal segments of various length and orientation (e.g., direct or inverted with respect to each other); and direct repeated segments of target DNA. The inverted terminal repeats present on most transposons allow for formation of stem-and-loop or hairpin-loop structures under the electron microscope, following denaturation and inter- or intra-strand reannealing (Kopecko, 1980).

Because they are relevant to this study, brief mention will be made of Class I and Class II transposons. Although similar in many respects, the major differences between Class I and Class II transposons are the length of the terminal repeats and the location and regulation of the genes involved in transposition. Class I transposons (composite elements) have long terminal repeats [about 786 to 1800 base pairs (bp)], that contain all of the information required for transposition. In fact, the ends of most Class I transposons are IS sequences, and therefore, are capable of transposition independent of the composite genome; one end is usually fully functional for providing transposition activity, while the other end is either totally defective or reduced in providing this activity (Berg et al., 1980; Kleckner, 1983). The inverted terminal repeats on Class II transposons are 35 to 40 bp in length, and do not encode for transposition activity (Heffron, 1983). Class II transposons share DNA sequence homology in the short terminal repeats, as well as throughout their entire length; very little DNA sequence homology exists among Class I transposons. Both
Figure 3. Structure of a typical transposon

The wavy lines denote central sequences that contain accessory genetic determinants, and in some instances (e.g., Kleckner Class II transposons) the information required for transposition. The repeated terminal sequences, varying in length from 38 base pairs (Class II) up to 768 to 1800 base pairs (Class I), are shown by the rectangles. Terminal repeat sequences can be in direct or indirect orientation. As a consequence of insertion, a 3 to 12 base-pair direct repeat of target DNA (broken lines) is duplicated on either side of the element. The target replicon is shown by thin continuous lines (taken from Kopecko, 1980).
TRANSPOSABLE DNA ELEMENT

- Flanking Duplicated Recipient DNA
- Central Sequences
- Terminal Repeat Sequences on Transposable Element
- Flanking Duplicated Recipient DNA

(Direct or Inverted)
the transposition and accessory genetic information are encoded in the central region of a Class II transposon. The two classes also differ with regard to the length of duplicated target DNA: Class I transposons generate a 3 to 12 bp duplication, and all Class II elements characteristically generate a 5 bp duplication of target DNA (Heffron, 1983; Kleckner, 1981). Transposition of composite Class I elements generates either precise transposition events, or relatively stable cointegrate structures (Kleckner, 1981). Class II elements transpose via a two-step mechanism; the donor and recipient replicons fuse concomitant with the duplication of the transposon, and then recombination between the two copies of the element yields a precise transpositional event (Kleckner, 1981). Finally, Class II elements exhibit cis-acting transposition immunity: a resident copy of a Class II transposon precludes further insertions of the same or a similar element onto the same replicon (Robinson et al., 1977; Wallace et al., 1980).

Transposons are valuable tools for genomic analysis. Tn elements are particularly useful for constructing strains and vectors, isolating mutants, providing portable regions of homology, selecting chromosomal duplications, chromosomal mapping, cloning genes, constructing gene fusions, and introducing well-defined deletions (Hille and Schilperoort, 1981; Kleckner, 1981; Kleckner et al., 1977; Luchansky and Pattee, 1984; Shapiro, 1982; Youngman et al., 1984a; Youngman et al., 1985a). Most transposons range in size from about 4 kb to 20 kb, and contain genes required for transposition, as well as accessory genetic information, such as genes involved in antibiotic resistance, heavy metal resistance,
carbohydrate metabolism, and toxin production (Calos and Miller, 1980; Cornelis et al., 1978; Kopecko, 1980; Schmitt et al., 1980; So et al., 1980). Transposition frequencies range from $10^{-4}$ to $10^{-7}$ per generation, and insertion into a structural gene causes insertional inactivation and can also cause polar effects on promoter-distal genes in an operon (Kleckner, 1981). The preference for an insertion site varies: transposons such as Tn554 (Krolewski et al., 1981; Phillips and Novick, 1979) and Tn7 (Lichtenstein and Brenner, 1982) demonstrate site-specific insertion, whereas most other elements can transpose to several sites on a particular replicon (Calos and Miller, 1980). For many Tn elements, transposition occurs via a duplication/transposition event: a copy of the element is present at both the original and new site (Kleckner, 1981). Recently, however, transposons Tn916, Tn554, and Tn10 have been shown to transpose via an excisive mechanism, resulting in loss of the element at the original site (Bender and Kleckner, 1986; Gawron-Burke and Clewell, 1984; Murphy and Lofdahl, 1984). For transposition of plant transposons, the excision/re-insertion mechanism is the rule rather than the exception (Saedler and Nevers, 1985).

The concept of selfish DNA (Orgel and Crick, 1980) has been applied to transposons: they do not exist to specifically contribute to the phenotype of the host organism, but transpose simply for perpetuation and dissemination purposes only. More recently, however, Tn elements were postulated to have an evolutionary role as mutator genes. Strains of E. coli harboring Tn5 or Tn10 displayed an advantage over otherwise isogenic strains when grown together in a chemostat (Biel and Hartl, 1983; Chao et
al., 1983). Tn10 conferred an advantage by increasing the mutation rate of the host bacterium. The IS elements of Tn10 were responsible for the mutator effect (Chao et al., 1983). The reader is referred to the following publications for a more thorough discussion of transposons and their mode of transfer: Bender and Kleckner, 1986; Berg et al., 1980; Bukhari, 1981; Casadaban et al., 1980; Foster et al., 1981; Grindley and Reed, 1985; Harshey et al., 1982; Heffron, 1983; Kopecko, 1980; Saedler and Nevers, 1985; Shapiro, 1980; and Shapiro, 1982).

Transposons in Gram-positive Bacteria

As mentioned previously, more is known about the genetic systems of Gram-negative bacteria (e.g., E. coli) than about Gram-positive systems (e.g., S. aureus). Furthermore, most Gram-negative systems are replete with a diverse set of well-characterized Tn elements, whereas relatively few Gram-positive elements have been identified and characterized. Until recently, only two phenotypically distinguishable classes of transposons were recognized in Gram-positive systems: a tetracycline-resistant transposon (Tn916), and three erythromycin-resistant transposons (Tn554, Tn551, and Tn917). Because Tn917 was the only transposon available for use in B. subtilis, De Lencastre et al., (1983) employed a Gram-negative transposon, Tn1000, in E. coli to mutagenize B. subtilis DNA cloned onto the shuttle plasmid pHM2; approximately 23% of the inserts recovered in pHM2 were in B. subtilis DNA. Upon transfer of pHM2::Tn1000 into B. subtilis, two insertions of Tn1000 were identified that affected the spoIIA gene (De Lencastre et al., 1983). As discussed below, the identification of additional Gram-positive transposons obviated the use of Gram-negative
transposons for mutagenesis and analysis of Gram-positive genes. Before providing more detailed information on the Tn elements essential to this study (Tn551, Tn4001, and Tn917), brief mention will be made of the other transposons available for genetic analysis in Gram-positive systems.

Besides Tn916 and Tn917, several other streptococcal transposons have been identified: Tn918, Tn925, and Tn3871 in Streptococcus faecalis; Tn919 in Streptococcus sanguis; and Tn1545 in Streptococcus pneumoniae. Tn916, Tn918, Tn919, and Tn925 all encode for resistance to tetracycline, Tn1545 encodes for aminoglycoside and MLS (macrolide, lincosamide, and streptogramin B) resistance, and transposons Tn917 and Tn3871 encode for inducible erythromycin resistance. Tn916, a 15.0 kb transposon first reported by Franke and Clewell (1980), has been employed for genomic analysis in several Gram-positive organisms (Gawron-Burke and Clewell, 1982; Ike et al., 1983; Ivins et al., 1986; Nida and Cleary, 1983). In addition to the excision/re-insertion mechanism for transposition, the conjugal behavior of Tn916 also distinguishes this element from other transposons; transfer of Tn916 was demonstrated in membrane-filter conjugations in the absence of detectable plasmid DNA (Clewell, 1981; Guild et al., 1982).

More recently, three related tetracycline-resistance transposons, all approximately 15 kb in length, were described in Streptococcus: Tn918 (Clewell et al., 1985); Tn919 (Fitzgerald and Clewell, 1985); and Tn925 (Korman et al., 1986). All of the tetracycline-resistant streptococcal transposons are similar with respect to size and restriction pattern to the prototype element, Tn916. Although Tn919 is also a conjugal transposon, it
differs from Tn916 with respect to an internal HincII fragment (Fitzgerald and Clewell, 1985). DNA hybridization studies demonstrated that Tn916 and Tn918 share strong sequence homology (Clewell et al., 1985). Tn916, Tn918, and Tn919 are unstable in E. coli; in the absence of tetracycline selection, all three elements are lost from the vector (Clewell et al., 1985; Fitzgerald and Clewell, 1985; Gawron-Burke and Clewell, 1984). Tn925 also transferred as a conjugal element from a chromosomal locus in B. subtilis to Streptococcus faecalis (Korman et al., 1986).

Banai and LaBlanc (1984) described a 5.1 kb transposon (Tn3871) in S. faecalis that encoded for inducible resistance to erythromycin and was similar in restriction digest pattern to Tn917. The 18.5-kb element Tn1545 (Carlier and Courvalin, 1982) was the first Gram-positive transposon operational in a Gram-negative bacterium (E. coli). Gawron-Burke and Clewell (1984) demonstrated low frequency transposition of Tn916 in E. coli under special circumstances. Most recently, the Gram-positive transposons Tn917 and Tn4430 (see below) were also shown to transpose in a Gram-negative host (Kuramitsu and Casadaban, 1986; Lereclus et al., 1986).

Several transposons have been described in S. aureus. Phillips and Novick (1979) identified Tn554 as a 6.9-kb repressor-controlled, site-specific element, encoding for spectinomycin resistance, as well as for resistance to erythromycin [(ermA); Murphy et al., 1981]. As mentioned previously, Tn554 transposes via the excision/re-insertion mechanism, and has not been associated with detectable extrachromosomal DNA (Murphy and Lofdahl, 1984; Wyman et al., 1974). Two novel features of Tn554, determined from DNA sequence analysis of Tn554/target junctions, are: 1)
the absence of direct or indirect terminally repeated DNA; and 2) the absence of duplicated target DNA (Murphy and Lofdahl, 1984; Murphy et al., 1985). Townsend et al. (1984) described the 5.2-kb gentamicin resistant transposon, Tn3851. Although hybridization studies have not yet been performed, Tn3851 is believed to be similar to the other S. aureus gentamicin element, Tn4001, because Tn3851 also shows cross resistance to tobramycin and kanamycin, and transposes to both chromosomal and plasmid loci (Townsend et al., 1984). Two apparently different penicillin-resistant S. aureus transposons have been described: a 7.3-kb element Tn3852 (Kigbo et al., 1985), and a 6.6-kb element Tn4201 (Goering and Weber, 1985). Trees and Iandolo (1986) reported the discovery and preliminary characterization of Tn4291, a 5.2-kb methicillin-resistance transposon in S. aureus. Tn4291 transposes to multiple, although limited, insertion sites; a primary insertion site bordering segments 10 and 11 on the circular map of S. aureus NCTC 8325 (see Luchansky and Pattee, 1984), and a secondary insertion site on plasmid pI524 (within the blaz gene) [David Trees, personal communication].

Most recently, Lereclus et al. (1986) identified a transposon, designated Tn4430, in B. thuringiensis. Tn4430 is related in structure and transposition behavior to the Kleckner Class II elements: Tn4430 generates a 5-bp duplication of target DNA, and contains identical 38-bp inverted terminal repeats similar in sequence to the ends of Tn3 (Lereclus et al., 1986). Other than genes required for transposition, no additional information (e.g., accessory genetic determinants) has yet been associated with Tn4430.
The Gram-positive transposon, Tn551, occurs naturally on plasmid pI258 in S. aureus, and transposes to various chromosomal and plasmid sites at a frequency of $10^{-4}$ to $10^{-5}$ events per donor genome (Luchansky and Pattee, 1984; Pattee et al., 1977; Novick et al., 1979a, 1979b). Tn551 is 5.2 kb in length, and contains the \textit{ermB} gene encoding for constitutive resistance to erythromycin. As a Class II transposon, Tn551 harbors terminal inverted repeats 40 bp in length, and duplicates 5 bp of target DNA as a consequence of insertion. Through DNA sequence analysis, Khan and Novick (1980) determined that the ends of Tn551 were similar to those of Tn\textsubscript{3} (e.g., fourteen of the first 18 nucleotide pairs of both transposons were identical). Furthermore, Tn551 and Tn917 share extensive DNA sequence homology throughout their entire lengths (Perkins and Youngman, 1984; Shaw and Clewell, 1985). As mentioned previously, Kleckner Class II elements share high DNA sequence homology, and a common origin has been proposed to account for the conservation of base pairs among the Class II transposons Tn3, Tn551, and Tn917 (Khan and Novick, 1980; Kleckner, 1981; Perkins and Youngman, 1984).

Restriction endonuclease maps of plasmid pI258 and Tn551 have been published (Novick et al., 1979a, 1979c). The use of Tn551 for genomic analysis was facilitated by the isolation of a thermosensitive mutation in the replication origin of plasmid pI258 (Novick, 1974). Briefly, cells containing pI258 are grown under non-permissive conditions in the presence of erythromycin to eliminate the plasmid and to select for chromosomal transposition events (Luchansky and Pattee, 1984; Pattee, 1981). Tn551 is
used extensively for cloning and mapping genes of interest in *S. aureus* (Berger-Bachi, 1983; Breidt and Stewart, 1986; Luchansky and Pattee, 1984; Pattee, 1981; Recsei et al., 1986; Schroeder and Pattee, 1984; Stahl and Pattee, 1983a, 1983b).

**Tn4001**

Lyon et al. (1984) reported the discovery of Tn4001, a 4.7-kb transposon encoding for resistance to gentamicin, tobramycin, and kanamycin (Gm<sup>R</sup>, Tm<sup>R</sup>, Km<sup>R</sup>). The 1.35-kb terminal inverted repeats on this element identify Tn4001 as a Kleckner Class I transposon. Tn4001 was originally detected in plasmid pSK1 from a multiresistant clinical isolate of *S. aureus* from Australia (Lyon et al., 1984). pSK1 is a 27.0-kb plasmid which also encodes for resistance to trimethoprim (Tp<sup>R</sup>), ethidium bromide (Eb<sup>R</sup>), and quaternary ammonium compounds [(Qa<sup>R</sup>); Lyon et al., 1984; Tennent et al., 1985]. Tn4001 transposes at a frequency of 10<sup>-4</sup> between plasmid and chromosomal sites (Lyon et al., 1984; Tennent et al., 1985). Although an extensive restriction map and DNA sequence for the element are not available, it is known that a 2.5-kb *HindIII* fragment carries the complete gentamicin resistance determinant [(the *aacA-aphD* gene); Lyon and Skurray, 1987; Lyon et al., 1984; Tennent et al., 1985]. As mentioned previously, Tn4001 is similar to the gentamicin-resistant transposon Tn3851 (Townsend et al., 1984). Insertions of Tn4001 have been isolated on the temperature-sensitive delivery vehicles pI258 and pII147 and in several regions of the *S. aureus* 8325 chromosome (Mahairas et al., manuscript in preparation).
Tn917

The erythromycin resistance transposon Tn917 was initially detected on plasmid pAD2 in *S. faecalis* strain DS16 (Tomich et al., 1978, 1980). Both erythromycin resistance and transposition of Tn917 are erythromycin inducible (Clewell et al., 1982; Perkins and Youngman, 1984). Although transposition of an *E. coli* mercury resistance transposon (Tn501) is enhanced by exposure to mercury ions, Tn917 is the only element that undergoes antibiotic-induced transposition (Clewell et al., 1982; Shaw and Clewell, 1985). The entire DNA sequence (5,257 base pairs) of Tn917 has been determined (Shaw and Clewell, 1985). Transcription and DNA-sequence analyses revealed that induction of MLS^E and transposition are coupled; transcriptional readthrough of a termination site at the end of the erm gene extends the mRNA transcript into the transposase gene (Shaw and Clewell, 1985). The actual induction of Tn917 MLS^E, however, is a posttranscriptional event that involves translational attenuation of the mRNA transcript, similar to the mechanism reported for induction of the ermC gene of pE194 and the erm gene of plasmid pAM77 (Horinouchi et al., 1983).

The ends of Tn917 were shown by DNA sequence analysis to be similar to the ends of other Kleckner Class II elements such as Tn3 and Tn551 (Perkins and Youngman, 1984; Shaw and Clewell, 1985). As mentioned previously, Tn917 shares extensive DNA sequence homology with transposons Tn551 and Tn3871 throughout the entire length of the element. It is thought that Tn917-like elements are widespread in *S. faecalis* (Rollins et al., 1985; Shaw and Clewell, 1985). Tn917 has been utilized extensively for genomic
analysis in *B. subtilis* (Vandeyar and Zahler, 1986; Youngman, 1985; Youngman et al., 1983), *S. faecalis* (Clewell et al., 1982; Ike and Clewell, 1984), and *S. aureus* (C. Hasegawa, Department of Microbiology, Iowa State University, Ames, Iowa, personal communication; J. B. Luchansky, Department of Microbiology, Iowa State University, Ames, Iowa, unpublished data).

Recently, Tn917 was successfully introduced and demonstrated to transpose in *B. amyloliquefaciens* (Hartley and Paddon, 1986), *B. megaterium* (Bohall and Vary, 1986), and *B. thuringiensis* (Crawford et al., 1986). Transposition of Tn917 has also been demonstrated in *E. coli*. Tn917 was transposed from a *B. subtilis*-*E. coli* shuttle vector into an F' plasmid, and then from the F' plasmid onto pACYC184 (Kuramitsu and Casadaban, 1986).

As described below, several novel plasmid vectors were constructed for recovery and utilization of Tn917 transpositions in Gram-positive bacteria.

Figure 4 contains a restriction map of Tn917, and shows the location of all 6 open reading frames (ORFs) of the element. ORF2 was identified as the region of Tn917 that encoded for the methylase that is responsible for conferring resistance to erythromycin. The *erm* gene of Tn917 differs by only four base pairs from the *erm* gene of plasmid pAM77 (Shaw and Clewell, 1985). A small control peptide (36 amino acids in length) encoded from ORF1 is believed to participate in the translational attenuation control of methylase synthesis (Shaw and Clewell, 1985). Using insertion and deletion mutagenesis, Perkins and Youngman (1984) identified a segment of Tn917 that was essential for transposition; deletion of a 1.3-kb HindIII fragment from Tn917 precluded transposition. The region of Tn917 encoding for the putative transposase is contained in ORF5. More recent evidence suggested
Figure 4. Diagram of Tn917

Tn917 is a 5,257 base-pair transposon that encodes for resistance to erythromycin. The orientation and location of the characteristic short (38 base pair) left and right inverted repeats, and an internal 38 base pair repeat, are designated as LR, RR, and IR, respectively, and are depicted by the thick arrows. The location of six likely open reading frames (ORF) are shown by the small open rectangles. Key restriction sites and position numbers for base pairs are delineated on the large rectangle. Although not clearly shown in the diagram, the IR is almost completely within the 3' end of ORF3 (taken from Shaw and Clewell, 1985).
that the gene product encoded by ORF6 may also be required for
transposition of Tn917 (Shaw and Clewell, 1985). DNA sequence analysis of
Tn917 revealed that ORF4 shared significant homology (30%) with the
recombinase gene of Tn3. The occurrence of a Tn3-like res site between
ORF3 and ORF4 further supports the role of the ORF4 gene product as a
recombinase (Shaw and Clewell, 1985). Finally, Tn917 harbors a 38-bp
internal repeat (IR), located between ORF3 and ORF4, identical in sequence,
and in the same orientation, as the left terminal repeat (LR) of the
transposon (see Figure 4). The flanking of the erm gene of Tn917 by IR and
LR creates an internal minor transposon present within a larger element. A
similar situation exists within the Kleckner Class II element, Tn1721
(Schmitt et al., 1980).

Several plasmid vectors have been constructed to utilize Tn917 in B.
subtilis, and other Gram-positive organisms, for both mapping and cloning
genes of interest (Youngman, 1985; Youngman et al., 1984c; Youngman et al.,
1985b). Many of these vectors are variations of plasmid pTV1, a 12.4-kb
plasmid that contains a temperature-sensitive origin of replication (from
plasmid pE194), and genes encoding for chloramphenicol resistance [(Cm\(^r\)];
cat gene from S. aureus plasmid pC194) and inducible erythromycin
resistance [(Em\(^r\)]; Tn917] (Youngman et al., 1983). A physical-genetic map
of pTV1 is given in Figure 5. Plasmid pE194 is a 3.7-kb multicopy plasmid
originating from S. aureus (Gryczan et al., 1982). When maintained in B.
subtilis, plasmid pE194 displays a progressively decreasing copy number
with increased temperature; replication of pE194 does not occur at
temperatures of 45 C or greater (Youngman, 1985).
Figure 5. Physical and genetic map of pTV1

Plasmid pTV1 is a 12.4 kb plasmid containing a temperature-sensitive origin of replication derived from *S. aureus* plasmid pE194 (pE194rep), and genes encoding for chloramphenicol resistance (*cat* gene from *S. aureus* plasmid pC194) and erythromycin resistance (*erm* gene of transposon Tn917 from *Streptococcus faecalis*). Arrows over the drug-resistance genes show the direction of transcription. The location of Tn917 is denoted by the large rectangle on the right-hand portion of the circle. The darkened box on each end of Tn917 delineates the 38 base pair terminal repeats. Kilobase pair coordinates are given on the inner arc of the circle, and the location of key restriction sites are shown on the outer arc of the circle. Closed circles (†) position *TaqI* cleavage sites and open boxes (‡) position *SauIII(MboI)* cleavage sites. The order of the closely spaced pair of *Aval*-*NcoI* cleavage sites, in parentheses, was not determined (taken from Perkins and Youngman, 1984).
Plasmid pTV1 was utilized as a temperature-sensitive delivery vehicle for isolating chromosomal Tn917 insertions in B. subtilis; several silent, auxotrophic, and insertional sporulation mutants of Tn917 were recovered at a frequency of 2 to 8 x 10^-5 (Youngman et al., 1983). When a pTV1-containing population of cells is grown at 30 C in the presence of the appropriate antibiotics, pTV1 is maintained as an autonomous replicon; however, when the temperature is shifted to at least 38 C, pTV1 is lost from this population of cells. If erythromycin is included in the medium when the cells are grown at a non-permissive temperature, erythromycin-resistant cells are recovered that no longer harbor autonomous pTV1, but contain a chromosomal copy of Tn917. Plasmid pTV1 has been used in this manner as a temperature-sensitive Tn917 delivery vehicle in various host strains (see above). As described below, pTV1 is used in this study as an insertion vector in S. aureus.

Youngman et al. (1984b) identified a unique HpaI restriction site within Tn917 in a region not affecting expression of erythromycin resistance or transposition functions (approximately 300 base pairs from the erm-proximal end of the element). To further extend the utility of this cloning site within a non-essential region of Tn917, the HpaI site was replaced by a 30 bp fragment of DNA containing BamHI and SmaI sites (Youngman et al., 1984b). Fragments of DNA [blunt-ended restriction fragments, or fragments generated by restriction enzymes in the BamHI/BclI/BglII/MboI(Sau3A) family] up to 4.5 kb in length have been inserted into this modified cloning site without affecting transposition (Youngman et al., 1985a). Genes encoding for resistance to thiostrepton or
chloramphenicol were inserted into the cloning site of Tn917, conferring new phenotypes on Tn917 (Youngman et al., 1985a, 1985b). The substituted derivatives of Tn917 will be of value for organisms where the Em^{R} phenotype could not be used (e.g., various species of _Streptomyces_ which are naturally Em^{R}).

Other derivatives of Tn917 were constructed to recover chromosomal DNA surrounding an insertion of Tn917 within or near a gene of interest. Tn917/pBR322 hybrids (e.g., pTV20) were constructed, containing the ampicillin resistance determinant and origin of replication from pBR322, plus the _cat_ gene from pC194, inserted into the _SalI_ site near the center of Tn917 (Youngman et al., 1984a). Upon transformation of competent _B. subtilis_ containing a specific chromosomal insertion of Tn917 with linearized pTV20 (digested with an enzyme that does not cut within transposon sequences), the entire transposon (including the Gram-negative sequences inserted at the _SalI_ site) becomes integrated via homologous recombination between plasmid-borne and chromosomal copies of Tn917. Transformants containing integrated pTV20 are selected for on chloramphenicol-containing media. Thus, a Gram-negative replicon (pBR322) becomes incorporated into a specific chromosomal locus in a Gram-positive host. Chromosomal DNA is isolated from a Cm^{R} transformant, and then digested with an enzyme having a single recognition site within the Gram-negative portion of the integrated transposon (e.g., _EcoRI_ for integrated pTV20). The _EcoRI_ fragments of _B. subtilis_ chromosomal DNA containing integrated plasmid are ligated and transformed into _E. coli_; selection is made for resistance to ampicillin. In this manner, DNA adjacent to a
chromosomal insertion of Tn917 is rapidly and efficiently cloned into E. coli for further analysis (Youngman, 1985; Youngman et al., 1984a, 1984c).

Finally, several derivatives of Tn917 and pTVl were constructed for generating transcriptional gene fusions. A promoterless lacZ gene, furnished with a functional B. subtilis ribosome-binding site, was inserted into the HpaI site of Tn917. The lacZ-substituted derivatives of Tn917 were fully functional for transposition. Transposon-mediated gene fusions were generated when lacZ-substituted Tn917 was inserted in the proper orientation within a chromosomal gene (Youngman et al., 1985a, 1985b). A fusion of a chromosomal promoter to the lacZ gene results in the production of beta-galactosidase instead of the normal gene product, and permits detection and quantitation of changes in the expression of the chromosomal promoter via the easy and rapid assays available for measuring the activity of beta-galactosidase. For the study of temporally regulated genes, such as the sporulation genes of B. subtilis, promoterless cat and lacZ genes were tandemly inserted into the HpaI site of Tn917 (Tn917 cat-lac). Transpositional fusions of Tn917 cat-lac to sporulation promoters were identified by the production of beta-galactosidase and chloramphenicol acetyl transferase during later stages of growth, but not early vegetative growth (Youngman et al., 1985a, 1985b). The derivatives of Tn917 and pTVl described in this section are adaptable for use in Gram-positive organisms other than B. subtilis, and should prove invaluable for gaining better insight into the molecular biology and genomic organization of these bacteria.
Recombinant DNA Methodology Applied to *S. aureus*

Chang and Cohen (1974) first reported the successful cloning of DNA fragments from *S. aureus* into *E. coli*. EcoRI-generated fragments of the staphylococcal plasmid pI258 (Lindberg and Novick, 1973; Novick and Bouanchaud, 1971), encoding for resistance to penicillin, erythromycin, cadmium, and several other heavy metal ions, and the *E. coli* tetracycline-resistance plasmid pSC101 were ligated and transformed into *E. coli*. A hybrid plasmid, designated pSC112, that conferred resistance to both penicillin and tetracycline was obtained (Chang and Cohen, 1974). Plasmid-containing transformants were not recovered when either CCC or linearized pI258 DNA alone were transformed into *E. coli*. The recovery of pSC112 demonstrated that *S. aureus* DNA (Gram-positive) was expressed in *E. coli* (Gram-negative). It was later shown that plasmid DNA from *S. aureus* would also replicate and express in a heterologous Gram-positive host, *B. subtilis*: one Tc<sup>r</sup> (pT127) and four Cm<sup>r</sup> (pC194, pC221, pC223 and pUB112) plasmids were successfully transformed into competent cells of *B. subtilis* (Ehrlich, 1977). In this same study, two additional *S. aureus* plasmids, pS120 and pK545, would not transform competent *B. subtilis* to either streptomycin (Sm<sup>r</sup>) or kanamycin/neomycin (Km<sup>r</sup>/Nm<sup>r</sup>) resistance, respectively. It was suggested that these plasmids may fail to replicate and/or express in *B. subtilis*, or perhaps their detection was precluded by the selection procedure employed [(e.g., high rate of spontaneous host mutations to these antibiotics); Ehrlich, 1977].

In general, many Gram-negative genes are not expressed in Gram-positive hosts when introduced as passenger DNA on recombinant molecules;
however, many Gram-positive genes are expressed in Gram-negative and/or heterologous Gram-positive hosts (Kreft et al., 1982; Trieu-Cuot et al., 1985a). This observation indicates that the process of transcription in Gram-positive bacteria is more stringent than the same process in Gram-negative bacteria. Recent evidence suggests that heterologous DNA is not expressed in *B. subtilis* because the $\sigma^{55}$ factor (now called $\sigma^{43}$) of *B. subtilis* RNA polymerase does not recognize the promoter region of these genes [(e.g., deviations from the *B. subtilis* -35 and -10 consensus sequence); Kreft et al., 1983]. It should be noted, however, that a few Gram-negative genes are expressed in a Gram-positive host (Ozaki et al., 1984; Trieu-Cuot et al., 1985b). In contrast, the major obstacles to acquisition of Gram-positive genes by a Gram-negative host are the transfer and replication of the foreign DNA (Trieu-Cuot et al., 1985b).

Another native *S. aureus* plasmid, pUB110 (2.8 md; Nm$^R$), was transformed into competent cells of *B. subtilis*, *B. pumilis*, and *B. licheniformis* (Keggins et al., 1978). pUB110 was utilized as a vector for cloning DNA fragments for complementation analysis of the trpC2 locus in *B. subtilis* (Keggins et al., 1978), and for cloning the alpha-amylase gene from *B. amyloliquefaciens* into *B. subtilis* (Palva, 1982). Noguchi et al. (1983) utilized deletion derivatives of another *S. aureus* plasmid, pTP-5 (2.9 md; Tc$^R$), in *B. subtilis* to define the regions of pTP-5 essential for autonomous replication and tetracycline resistance. Ehrlich et al. (1982) and Gryczan et al. (1978) reviewed the various plasmids of *S. aureus* origin that have been used for cloning in *B. subtilis*. Furthermore, *S. aureus* plasmids (or derivatives thereof) have been successfully introduced and
subsequently maintained in such phylogenetically distant organisms as *B. megaterium* (Brown and Carlton, 1980), *B. thuringiensis* (Martin et al., 1981), *E. coli* (Goze and Ehrlich, 1980), *Saccharomyces cerevisiae* (Goursot et al., 1982), and *Streptococcus pneumoniae* (Barany et al., 1982).

Following the discovery that *S. aureus* plasmid DNA would replicate and express in heterologous hosts (Barany et al., 1982; Chang and Cohen, 1974; Ehrlich, 1977; Goze and Ehrlich, 1980; Keggins et al., 1978; Noguchi et al., 1983), several investigators characterized and developed indigenous staphylococcal plasmids for the manipulation of recombinant DNA in *S. aureus* (Iordanescu, 1975; Lofdahl et al., 1978a, 1978b; Wilson and Baldwin, 1978). The majority of these plasmids were small (less than 5 md) tetracycline- or chloramphenicol-resistance plasmids with unique restriction sites for EcoRI and/or HindIII (Lofdahl et al., 1978a; Wilson and Baldwin, 1978). One such recombinant plasmid, pSC194 (Cm<sup>R</sup>Sm<sup>F</sup>), was constructed in vivo by cotransduction of *S. aureus* plasmids pS194 (Sm<sup>F</sup>) and pC194 (Cm<sup>F</sup>); 5 to 10% of the transductants recovered by the selection for one plasmid had also acquired the other unselected plasmid and its associated marker (Iordanescu, 1975). Lofdahl et al. (1978a) characterized all three plasmids with respect to size, restriction digest pattern, and transforming activity. Plasmids pC194, pS194, and pSC194 are 2, 3, and 4.9 md in size, respectively. All three plasmids yielded about 1000 transformants/ug CCC DNA when transformed into competent cells of *S. aureus*; a linear relationship was obtained between DNA concentration and the number of transformants. Linearized *S. aureus* plasmid DNA exhibited a 10-fold lower frequency of transformation for competent cells of *S. aureus*.
compared to CCC DNA of the same plasmid. Restriction endonuclease analysis revealed that pS194 contained unique sites for EcoRI and HindIII, pC194 a single HindIII site, and pSC194 a single EcoRI and two HindIII sites. In addition, CCC DNA of pC194 and pSC194 transformed competent cells of B. subtilis to Cm^R at a frequency of 100 Cm^R transformants/ug CCC DNA (Ehrlich, 1977; Lofdahl et al., 1978a).

Plasmid pSC194 was proposed as a suitable cloning vector for both S. aureus and B. subtilis. B. subtilis was envisioned as an alternative host to E. coli for recombinant DNA manipulations involving Gram-positive genes. Plasmid pSC194 was utilized to clone fragments of staphylococcal plasmid pI258 to develop a physical map for pI258 (Lofdahl et al., 1978b). The str gene of pSC194 harbors a single EcoRI site; therefore, recombinant clones were readily selected by screening for Cm^R Sm^R transformants. The blaZ gene (Saunders et al., 1984a), the ermB gene (Lofdahl et al., 1978b; Wilson and Baldwin, 1978), and the genes responsible for resistance to mercury (merA and merB) and cadmium [(cadA and cadB); Witte et al., 1986], all from plasmid pI258, have been cloned. The components of plasmid pI258 were cloned to analyze the expression of blaZ in a heterologous host (B. subtilis), to develop a physical map for pI258, and to determine the relationship between chromosomal and plasmid-borne mercury or cadmium resistance determinants.

Both pC194 and derivatives of pC194 have been utilized as cloning vehicles in B. subtilis (Canosi et al., 1981; Ehrlich, 1978). In these studies, monomeric forms of hybrid plasmids composed of pC194 and B. subtilis chromosomal DNA were active in transformation of competent,
recombination-proficient cells of *B. subtilis*: monomeric pC194 without an insert of *B. subtilis* passenger DNA did not transform *B. subtilis* (Canosi et al., 1981). *S. aureus* cloning vectors have been developed with EcoRI, HindIII, BglII, and PstI cloning sites (Wilson and Baldwin, 1978). The cloning sites on these vectors were not within the structural genes of the antibiotic resistance determinants; therefore, rapid screening for successful clones by gene inactivation was not possible with these vectors. Furthermore, several of the plasmids described above were unstable in certain hosts (Grandi et al., 1981; Kreft et al., 1982; Primrose and Ehrlich, 1981). Wilson et al. (1981) improved upon several of these vectors by incorporating additional selectable markers, as well as unique restriction sites within these determinants for insertion of passenger DNA (gene inactivation). Keller et al. (1983) constructed and characterized insertional inactivation vectors (e.g., pCT20 and pCE10) that circumvented plasmid instability for cloning in *S. aureus* and *S. carnosus*.

The demonstration that *S. aureus* DNA was replicated and expressed in heterologous hosts, along with the development of suitable cloning vectors, resulted in the cloning and/or DNA sequencing of various *S. aureus* genes. The gene for protein A (*spa*) was independently cloned by Duggleby and Jones (1983) and Lofdahl et al. (1983). A complete nucleotide sequence for the *spa* gene has been reported (Uhlen et al., 1984). Shortle (1983) successfully cloned the gene encoding for staphylococcal nuclease (*nuc*) into *E. coli*. Two genes encoding for extracellular proteins involved in staphylococcal food poisoning have been cloned: staphylococcal enterotoxin A [(SEA); *entA*; Betley and Mekalanos, 1985; Betley et al., 1984]; and
staphylococcal enterotoxin B [(SEB); entB; Ranelli et al., 1985]. Jones and Khan (1986) recently published the nucleotide sequence of the entB gene. Other genes of interest from S. aureus that have been cloned and/or sequenced include, staphylokinase (Sako et al., 1983), the alpha hemolysin structural gene (hly; Fairweather et al., 1983; Kehoe et al., 1983), the gene for toxic shock syndrome toxin (tst; Kreiswirth et al., 1983), a beta-lactamase gene (blaZ; Saunders et al., 1984a), the exfoliative toxin B structural gene (etb; Jackson and Iandolo, 1986a, 1986b), and the lipase structural gene (geh; Lee and Iandolo, 1985; Lee and Iandolo, 1986). It should be noted, however, that molecular cloning as an approach to chromosomal analysis of S. aureus has been largely overlooked.

### Chromosomal Mapping in S. aureus

Genetic analysis in S. aureus NCTC 8325 has been conducted extensively by transformation, and to a lesser degree by transduction. Beginning in the late 1950s and continuing to the present, several investigators have progressively developed generalized transduction into an invaluable means for fine structure analysis within defined regions of the S. aureus chromosome (Kloos and Pattee, 1965; Pattee et al., 1974; Proctor and Kloos, 1970; Ritz and Baldwin, 1962; Schroeder and Pattee, 1984). Lindberg et al. (1972) demonstrated that S. aureus can become competent for the uptake of naked DNA (e.g., was amenable to transformation). Pattee and Neveln (1975) utilized transformation to arrange available markers into three distinct linkage groups. The linkage groups were further expanded by the isolation, and subsequent mapping via transformation, of several additional genetic
determinants, including mec-4916, pig-131, and purB110 (Kuhl et al., 1978; Pattee, 1976).

Sufficient loci were defined for Pattee and Glatz (1980) to divide each of the three linkage groups into segments that overlapped; each segment was bordered by a pair of markers that were co-transformable. The size and detail of the map was expanded by using Tn551 mutagenesis to recover auxotrophic and silent insertions of Tn551 in different regions of the S. aureus chromosome (Pattee, 1981). A procedure for protoplast fusion analysis of S. aureus chromosomal markers was developed that predicted the relative orientation of all three linkage groups (Stahl and Pattee, 1983a, 1983b). Transformation with DNA of high molecular weight confirmed the order of markers predicted by protoplast fusion as the correct permutation, and resulted in a circular (though noncontiguous) chromosomal arrangement of genetic determinants (Stahl and Pattee, 1983b). Several chromosomal determinants could not be mapped, however, because they did not occupy sites within or near any of the existing linkage groups, and/or because it was not possible to directly select for these markers. To circumvent these problems, efforts were directed to isolate chromosomal insertions of Tn551 near markers of interest to define their location relative to known markers and extend the limits of the existing linkage groups (Luchansky and Pattee, 1984).

Recently, a conjugation system was developed for genomic analysis of S. aureus (Stout, 1986). A chromosomal copy of Tn551 provided homology to a copy of Tn551 present on a conjugal gentamicin-resistance plasmid that promotes chromosome mobilization. Mobilization of genes from either side
of the chromosomal insertion occurred, but stable F'-like plasmids were not observed (Stout, 1986). In the present study, a novel integrable plasmid (derived from plasmid pTV1ts) was constructed to thoroughly exploit chromosomal transposon insertions for analyzing the genome of S. aureus.
MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The cloning vector-insertion plasmid pJH101 was kindly provided by Dr. James Hoch. pJH101 was constructed by inserting the chloramphenicol acetyl transferase (cat) gene from pCl94, a 2.0-megadalton (md) S. aureus plasmid, into the unique PvuII site of the E. coli plasmid pBR322. pJH101 does not contain an origin of replication for autonomous maintenance in a Gram-positive host, but the cat gene is expressed in both E. coli and B. subtilis: pJH101 replicates in E. coli by using the pBR322 origin of replication. The E. coli vectors pJH101 (Ferrari et al., 1983) and pBR322 (Bolivar et al., 1977), as well as the S. aureus plasmid pCl94 (Horinouchi and Weisblum, 1982b), have been described previously. Plasmids pI258 and pPQ58 encode for resistance to erythromycin (Tn551), as well as resistance to several heavy metal ions, and for production of a beta-lactamase. Plasmid pI258 has been described previously (Novick, 1974; Novick et al., 1979c). pI258, which contains an insertion of the gentamicin-resistance transposon Tn4001, is referred to as plasmid pPQ58 (pPQ58 was obtained from Gregory G. Mahairas, Department of Microbiology, Iowa State University, Ames, Iowa).

Dr. Phillip J. Youngman kindly provided strains of B. subtilis containing plasmids pTV20 and pTV1\textsubscript{ts}. pTV20 was constructed by insertion of the pBR322-derived replicon pHW9 into a SalI restriction site near the middle of Tn917; pTV20 has been used to clone sequences adjacent to chromosomal Tn917 insertions (Youngman et al., 1984a). Cesium chloride
Table 1. Designation, genotype, and origin of strains of *Staphylococcus aureus*

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<tr>
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<td>8325nov-142 pig-131</td>
<td>Pattee and Neveln, 1975</td>
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<td>8325rvh-101 thrB106 ilv-129 pig-131</td>
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<td>8325-4pig-131</td>
<td>Thompson and Pattee, 1977</td>
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<td>ISP130</td>
<td>8325-4[pI258blaI443 asa-33 ermB20 repA18]500 pig-131</td>
<td>Schwesinger and Novick, 1975</td>
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<td>Stobberingh^a</td>
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<td>ISP459</td>
<td>8325r1^- m31+ r2^- m32+ pig-131</td>
<td>Iordanescu and Surdeanu, 1976</td>
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<td>ISP479</td>
<td>8325-4(pI258 bla-401 mer-14 repA36) pig-131</td>
<td>Pattee, 1981</td>
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<td>ISP778</td>
<td>80CR3nov-142</td>
<td>80a/ISP2 X ISP456^b</td>
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<td>ISP794</td>
<td>8325pig-131</td>
<td>Stahl and Pattee, 1983b</td>
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<td>8325pig-131 w[Chr::Tn551]34</td>
<td>Stahl and Pattee, 1983a</td>
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<td>8325-4trp-159::Tn551 ermB317 pig-131</td>
<td>Pattee et al., 1983</td>
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<td>ISP856</td>
<td>8325-4(pC194)</td>
<td>RN2425^c</td>
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^a Ellen Stobberingh, Biomedical Centre, State University Limburg, Maastricht, The Netherlands.

^b ISP456 was transduced with a phage 80a lysate prepared on ISP2. The methods have been described (Schroeder and Pattee, 1984).

^c Richard P. Novick stock culture collection, Department of Plasmid Biology, the Public Health Research Institute of the City of New York, Inc., New York, NY.
Table 1 (Continued)

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<td>ISP459 X ISP849(UV)&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Stahl and Pattee, 1983b</td>
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<td>RN981&lt;sup&gt;c&lt;/sup&gt;; Wyman et al., 1974</td>
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<td>ppISP1046 X ISP1479&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>80a/ISP1505 X ISP8</td>
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<td>(p1258 bla&lt;sup&gt;1401&lt;/sup&gt; mer-14 rep&lt;sup&gt;A36&lt;/sup&gt;)</td>
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</table>

<sup>d</sup>UV-irradiated ISP849 fused with protoplasts of ISP459.

<sup>e</sup>ISP997 was transformed with strain ISP796 DNA.

<sup>f</sup>ISP778 cured of Cd<sup>+</sup> plasmid by protoplast formation.

<sup>g</sup>Ronald A. Skurray, Department of Microbiology, Monash University, Clayton, Victoria, Australia.

<sup>h</sup>Protoplast fusion between ISP1046 and ISP1479 protoplasts.
Table 1 (Continued)

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^1ISP8 was transduced with an 80a lysate prepared on nitrosoguanidine mutagenized ISP1421.

^1ISP1506 grown at 43°C to cure pII147 from the culture.

^kGregory G. Mahairas, Department of Microbiology, Iowa State University, Ames, IA.

^lTransformant obtained from a ligation mixture of HindIII-digested, CIP-treated pTV1tS and HindIII-digested pPQ58 presented to protoplasts of ISP1658.
Table 1 (Continued)

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^aIsolate recovered following transformation of ISP990 with plasmid pTV1^a when selection was made for resistance to 10 μg/ml of chloramphenicol (JBL10, JBL11, JBL14a), or 1.5 μg/ml of erythromycin (JBL14b).

^bTransformant obtained following transformation of ISP5 with high molecular weight DNA extracted from a population of cells of ISP1846 previously grown at 43 C with appropriate selection. Contains a deleted derivative (Gm^a) of plasmid pPQ126 presumably integrated into the trp-159::Tn551 ermB317 target site of donor ISP1846 DNA. A Class D isolate from Table 18.
### Table 1 (Continued)

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°Transformant obtained following transformation of ISP5 with high molecular weight DNA extracted from a population of cells of ISP1846 previously grown at 43°C with appropriate selection. A Class F isolate (Table 18) with pPQ126 presumably integrated at the trp-159::Tn551 ermB317 target site of donor ISP1846 DNA.

^Transformant obtained following transformation of ISP5 with high molecular weight DNA extracted from a population of cells of ISP1846 previously grown at 43°C with appropriate selection. A Class A isolate (Table 18) with pPQ126 presumably integrated at the trp-159::Tn551 ermB317 target site of donor ISP1846 DNA.

^Transformation of ISP5 with DNA isolated from JBL85 grown at 43°C with selection (see Table 19).
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<td>JBL117</td>
<td>$8325-4r^{-} \ (pPQ126)$</td>
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$^r$Isolate from a BHI agar plate incubated at 30 C from a plasmid elimination experiment with JBL85.

$^s$Isolate from a BHI agar plate containing 10 ug/ml of erythromycin incubated at 30 C from a plasmid elimination experiment with JBL85.

$^t$Isolate from a BHI agar plate incubated at 39 C from a plasmid elimination experiment with JBL85.

$^u$Isolate from a BHI agar plate containing 10 ug/ml of erythromycin incubated at 39 C from a plasmid elimination experiment with JBL85.

$^v$Isolate from a BHI agar plate incubated at 30 C from a plasmid elimination experiment with ISP1844.

$^w$Isolate from a BHI agar plate containing 10 ug/ml of erythromycin incubated at 30 C from a plasmid elimination experiment with ISP1844.
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<td>ISP1801(^{bb})</td>
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\(^{x}\) Loopful of cells from a BHI agar plate containing 10 \(\mu\)g/ml of erythromycin incubated at 39 °C from a plasmid elimination experiment with ISP1844 (see Table 12).

\(^{y}\) Isolate from a BHI agar plate containing 10 \(\mu\)g/ml of gentamicin incubated at 30 °C from a plasmid elimination experiment with ISP1844.

\(^{z}\) Isolate from BHI agar plate incubated at 39 °C from a plasmid elimination experiment with ISP1844.

\(^{aa}\) Isolate from a BHI agar plate containing 10 \(\mu\)g/ml of gentamicin incubated at 39 °C from a plasmid elimination experiment with ISP1844.

\(^{bb}\) ISP1801 grown at 43 °C to cure pPQ61 from the culture.

\(^{cc}\) Isolate from a BHI agar plate incubated at 30 °C from a plasmid elimination experiment with JBL177.
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ddIsolate from a BHI agar plate containing 10 μg/ml of gentamicin incubated at 30 C from a plasmid elimination experiment with JBL177.

eeIsolate from a BHI agar plate containing 10 μg/ml of erythromycin incubated at 30 C from a plasmid elimination experiment with JBL177.

ffIsolate from a BHI agar plate incubated at 39 C from a plasmid elimination experiment with JBL177.

ggIsolate from a BHI agar plate containing 10 μg/ml of gentamicin incubated at 39 C from a plasmid elimination experiment with JBL177.

hhIsolate from a BHI agar plate containing 10 μg/ml of erythromycin incubated at 39 C from a plasmid elimination experiment with JBL177.
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\text{ii} Isolate from a BHI agar plate incubated at 30 C from a plasmid elimination experiment with ISP1846.

\text{jj} Isolate from a BHI agar plate incubated at 39 C from a plasmid elimination experiment with ISP1846.

\text{kk} Isolate from a BHI agar plate containing 10 ug/ml erythromycin incubated at 30 C from a plasmid elimination experiment with ISP1846.

\text{ll} Isolate from a BHI agar plate containing 10 ug/ml erythromycin incubated at 39 C from a plasmid elimination experiment with ISP1846.

\text{mm} Isolate from a BHI agar plate containing 10 ug/ml of gentamicin incubated at 30 C from a plasmid elimination experiment with ISP1846.
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<sup>nn</sup>Isolate from a BHI agar plate containing 10 ug/ml of gentamicin incubated at 39. C from a plasmid elimination experiment with ISP1846.

<sup>oo</sup>Isolate from a BHI agar plate incubated at 30 C from a plasmid elimination experiment with ISP1578.

<sup>PP</sup>Isolate from a BHI agar plate incubated at 39 C from a plasmid elimination experiment with ISP1578.

<sup>qq</sup>Isolate from a BHI agar plate containing 10 ug/ml of erythromycin incubated at 30 C from a plasmid elimination experiment with ISP1578.

<sup>rr</sup>Isolate from a BHI agar plate containing 10 ug/ml of erythromycin incubated at 39 C from a plasmid elimination experiment with ISP1578.
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<td>Boyer &amp; Roulland-Dussoix, 1969</td>
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<td>F&lt;sup&gt;-&lt;/sup&gt; had&lt;sup&gt;20&lt;/sup&gt; (r&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;m&lt;sub&gt;B&lt;/sub&gt;) ara-14</td>
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<sup>ss</sup>James A. Hoch, Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, CA.

<sup>tt</sup>Transformant obtained from transformation of pBR322 into E. coli HB101.

<sup>uu</sup>Transformant obtained from a ligation mixture of HindIII-digested pBR322 and the 1.67 md HinF fragment of plasmid pPQ58 presented to competent cells of ISP1196.

<sup>vvv</sup>Transformant obtained from a ligation mixture of HindIII-digested, CIP-treated pJH101 and the 2.8 md HinB fragment (erm<sub>B</sub> gene of Tn551) of plasmid pI258 presented to competent cells of ISP1196.
Table 1 (Continued)

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^W^Transformant obtained from a ligation mixture of HindIII-digested, CIP-treated pJH101 and HindIII-digested pI258 presented to competent cells of ISP1196.

^X^Transformant obtained from a ligation mixture of HindIII-digested pBR322 and HindIII-digested pI258 presented to competent cells of ISP1196.

^YYPhillip J. Youngman, Department of Microbiology, University of Pennsylvania, School of Medicine, Philadelphia, PA.
**Table 1 (Continued)**

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<td>Horinouchi and Weisblum, 1982b</td>
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<td>pE194</td>
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**Abbreviations:** **Ap**<sup>-</sup>, ampicillin; Bla<sup>+</sup>, constitutive beta-lactamase; Cd, cadmium; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; Tc, tetracycline; Tsr, temperature sensitive replication.

<sup>aaa</sup>Peter A. Pattee, Department of Microbiology, Iowa State University, Ames, IA.

<sup>bbb</sup>A recombinant plasmid composed of the 11.1 kb HindIII fragment of plasmid pTV1<sub>ts</sub> ligated to the 2.5 kb Gm<sup>-</sup> HindIII fragment of plasmid pPQ58.

<sup>ccc</sup>Deleted derivative of pTV1<sub>ts</sub> obtained following transformation of ISP990 with pTV1<sub>ts</sub> and selecting for resistance to 10 ug/ml chloramphenicol.
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**ddd** Derivative of pPQ126 obtained following transformation of ISP5 with high molecular weight DNA extracted from a population of cells of JBL85 previously grown at 43 C. All incubations and platings for the transformation were performed at 34 C, and selection was made for resistance to erythromycin (Table 19).

**eee** Plasmid(s) obtained by growing JBL113 (containing chromosomally integrated pPQ126) at 30 C in the presence of 3 ug/ml each of erythromycin and gentamicin and 1 ug/ml of chloramphenicol.

**fff** The 2.8 md HinB fragment (ermB gene of Tn551) of plasmid pI258 ligated into the unique HindIII site of plasmid pJH101.

**ggg** The HinG fragment of plasmid pI258 ligated into the unique HindIII site of plasmid pJH101.

**hhh** The 2.8 md HinB fragment (ermB gene of Tn551) of plasmid pI258 ligated into the unique HindIII site of plasmid pBR322. The ermB gene encodes for resistance to 300 ug/ml of erythromycin in E. coli HB101.

**iii** The 1.67 md HinF fragment (gentamicin-resistance determinant of Tn4001) of plasmid pPQ58 ligated into the unique HindIII site of plasmid pBR322. The gentamicin resistance determinant encodes for low-level resistance (at least 1 ug/ml) to gentamicin in E. coli HB101.
(CsCl) purified pTV20 DNA, isolated from B. subtilis, was provided by Dr. Peter A. Pattee. pTVl was constructed by ligating the Tn917-containing PvuII restriction fragment of pAM(alphal)(deletionl):Tn917 into plasmid pBD95 (Youngman et al., 1983). A highly temperature-sensitive derivative of pTVl (designated pTVlTS) was constructed that displays an abrupt replication block at 38 C (Youngman, 1985). pTVlTS is a 12.4 kb plasmid composed of 2 HindIII fragments (Youngman, 1985); a large 11.1 kb fragment contains the replication functions (pE194TS origin of replication), as well as genes encoding for resistance to chloramphenicol (cat gene from pC194) and erythromycin (erm gene of Tn917), and a smaller 1.3 kb fragment that contains information essential for transposition (Perkins and Youngman, 1984). pTVlTS has been used as a Tn917 delivery vehicle (Youngman et al., 1983). Nitrosoguanidine treatment of a population of cells containing pE194, a 3.7 kb erythromycin-resistance vehicle native to S. aureus (Horinouchi and Weisblum, 1982a), produced plasmid pE194TS (Youngman, 1985). Chromosomal DNA isolated from ISP1010 (control DNA for competent-cell transformation of S. aureus) was kindly provided by Dr. Peter A. Pattee. Cesium chloride purified pBR322 was generously provided by Dr. Kim Engwall. All of the strains used in this study are available from the ISP culture collection of Dr. Peter A. Pattee and/or the JBL culture collection of John B. Luchansky (Department of Microbiology, Iowa State University, Ames, Iowa).

Several strains in this study contain chromosomal insertions of the transposable elements, Tn917, or Tn551, or Tn4001. Following the recommendations of Novick et al. (1976) and Campbell et al. (1979), the
Greek letter omega (\(\omega\)), and the notation \([\text{Chr}::\text{Tn}\text{XXX}]\) followed by an isolation number, are used to denote silent insertions of Tn elements (e.g., \(\omega[\text{Chr}::\text{Tn}551]34\)). Insertions resulting in the inactivation of certain genes are identified by the gene symbol and allele number, followed by the notation ::Tn551 (e.g., \(\text{uraB232::Tn}551\)). Some strains carry a copy of Tn551 containing a mutation in the \(\text{ermB}\) gene that results in an erythromycin-sensitive phenotype (e.g., \(\text{uraB232::Tn}551\ \text{ermB327}\)). A detailed description of the isolation of \(\text{Em}^S\) mutants of Tn551 resulting from spontaneous point mutations within the \(\text{ermB}\) gene of this transposon has been provided (Pattie et al., 1983).

**Culture Media**

Except where noted, 1.5% (w/v) Bacto-agar (Difco Laboratories, Detroit, MI) was added to all of the commercially available dehydrated culture media used in this study to prepare both agar plates and slants (Brain Heart Infusion (BHI; Difco), and Trypticase Soy Broth (TSB; BBL, Cockeysville, MD)). Trypticase Soy Agar (BBL) containing chloramphenicol was used to select and score for resistance to chloramphenicol. Oxoid Tryptone Soya Broth (Oxoid TSB; Oxoid Ltd., Basingstoke, Hants, England) was used for growing cells of \(S. \text{aureus}\) to competence. For protoplast plasmid transformation experiments, cells were grown to the proper density in antibiotic medium 3 (Penassay broth; Difco), and putative transformants were plated onto either DM3 medium or Regeneration (R) agar. DM3 was prepared essentially as described by Chang and Cohen (1979): 500 ml sodium succinate (Sigma Chemical Co., St. Louis, MO; 1 M; pH 7.3), 20 ml MgCl\(_2\) (1 M), 5 ml Bovine Serum Albumin (BSA; Sigma; 2%), 25 ml of glucose (20%), 100
ml $K_2PO_4/KH_2PO_4$ solution (3.5% $K_2PO_4$ and 1.5% $KH_2PO_4$), 50 ml Bacto-yeast extract (yeast extract; Difco; 5%), and 100 ml casamino acids (Difco; 5%). The percent solutions for DM3 medium are listed on a weight per volume basis. All of the DM3 components were autoclaved separately, except for BSA (filter-sterilized) and sodium succinate (steam-sterilized with 200 ml of H$_2$O-agar (8 g]). R agar (Stahl and Pattee, 1983a) consisted of 273 g of sucrose (ultra pure; Schwarz/Mann Inc., Spring Valley, NY), 30 g TSB, 0.5 g sodium citrate, 3.0 g soluble potato starch (Sigma), and 25 g of agar, all per liter of deionized water (dH$_2$O). The final pH was adjusted to 7.3 with 1 N NaOH.

All media for culturing strains of S. aureus were routinely supplemented with thymine (20 ug/ml), and adenine, cytosine, guanine, and uracil (5 ug each/ml). Unless stated otherwise, all agar media were routinely supplemented with sodium citrate (Fisher Scientific Co., Fair Lawn, NJ) at 1 g per liter. All cultures of S. aureus and E. coli were stored at 4 C on BHI agar slants and Luria-Bertani (LB) agar slants, respectively. LB medium, modified from Maniatis et al. (1982), consisted of 10 g Bacto-tryptone (tryptone; Difco), 5 g yeast extract, and 5 g NaCl, all per liter of deionized water and adjusted to a pH of 7.3. Bacteria containing plasmids were maintained at the proper temperature in the presence of the appropriate antibiotic(s) (e.g., incubation at 30 C on BHI agar plates containing 3 ug/ml each of erythromycin and gentamicin and 1 ug/ml of chloramphenicol for pPQ126-containing cells) to insure against loss of extrachromosomal elements. A second set of stock cultures were maintained at -70 C in GL broth plus 10% glycerol. GL broth consisted of 3
66
g each of casamino acids and yeast extract, 5.9 g NaCl, 3.3 ml of 60% sodium lactate, and 4 ml of 25% glycerol, all in 1000 ml deionized water adjusted to a final pH of 7.8 (Novick et al., 1979b). The composition of Complete Defined Synthetic (CDS) agar (Pattee and Neveln, 1975), used for selection and scoring of nutritional markers, is listed in Table 2. Table 3 describes the precise formulations for the various media used in this study to select and score genetic markers, along with the phenotypes of these markers.

Reagents and Buffers

All antibiotics used in this study were purchased from Sigma, and stored at -10 C as stock solutions prepared at a concentration of 10 mg/ml, as follows: chloramphenicol and erythromycin were dissolved in 95% ethanol, and tetracycline, gentamicin, and ampicillin were dissolved in deionized water and filter sterilized. Lysostaphin (Sigma), for disrupting the integrity of S. aureus cell walls, was dissolved at 1 mg/ml in a solution containing 600 mg Tris(hydroxymethyl)aminomethane (Tris; Sigma), 870 mg NaCl, and 100 ml deionized water, pH 7.5. Following positive-pressure filtration, 1-ml aliquots were stored at -10 C. Lysozyme (Sigma) was directly added at a concentration of 4 mg/ml to cells of E. coli in lysis buffer. The lysis buffer for both S. aureus and E. coli consisted of 200 g sucrose, 18.6 g ethylenediamine-tetraacetic acid [disodium salt] (EDTA; Fisher Scientific Co.), 3.95 g Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; Sigma): all 3 reagents were added to 800 ml deionized water, the pH was adjusted to 8.0, and the final volume was brought to 1 liter. The lysis buffer was dispensed in 150-ml portions and
Table 2. Composition of Complete Defined Synthetic (CDS) agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ingredient</th>
<th>Amount&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose:</td>
<td>5 gm</td>
<td>amino acids:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-glutamic acid</td>
<td>100 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-serine</td>
<td>30 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-methionine</td>
<td>10 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-tyrosine</td>
<td>50 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-lysine</td>
<td>50 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-alanine</td>
<td>60 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-threonine</td>
<td>30 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-phenylalanine</td>
<td>40 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-histidine</td>
<td>20 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>glycine</td>
<td>50 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-tryptophan</td>
<td>10 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-isoleucine</td>
<td>30 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-valine</td>
<td>80 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-leucine</td>
<td>90 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-aspartic acid</td>
<td>90 gm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-arginine</td>
<td>50 gm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-proline</td>
<td>80 gm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-cystine</td>
<td>20 gm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacto-Agar (Difco)</td>
<td>15 gm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>deionized water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

<sup>a</sup>Synthetic media were prepared by combining required volumes of each stock; these mixtures were filter-sterilized and combined with the agar, which had been previously autoclaved (121 °C, 15 min) with the remaining water, and cooled to 50 °C.

<sup>b</sup>Final concentration per liter of Complete Defined Synthetic (CDS) medium.

<sup>c</sup>Added only when needed.
Table 3. Selectable genetic markers, their phenotypes, and composition of media used for their selection and scoring

<table>
<thead>
<tr>
<th>Markers</th>
<th>Phenotypes</th>
<th>Media Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn551</td>
<td>Em^r erythromycin</td>
<td>+1 ug/ml erythromycin for selection, or +10 ug/ml for scoring of unselected markers in BHI agar</td>
</tr>
<tr>
<td></td>
<td>sensitive mutation</td>
<td>(see Tn551, above)</td>
</tr>
<tr>
<td>ermB</td>
<td>Em^s erythromycin</td>
<td>(see Tn551, above, but sub, substitute gentamicin for erythromycin)</td>
</tr>
<tr>
<td></td>
<td>sensitive mutation</td>
<td>(see Tn551 above, but substitute gentamicin for erythromycin)</td>
</tr>
<tr>
<td>Tn4001</td>
<td>Gm^r gentamicin</td>
<td>+30 ug/ml ampicillin for selection or +50 ug/ml for scoring of unselected markers in LB agar</td>
</tr>
<tr>
<td></td>
<td>resistance</td>
<td></td>
</tr>
<tr>
<td>Tn917</td>
<td>Em^r erythromycin</td>
<td>+25 ug/ml tetracycline for scoring of unselected markers in LB agar</td>
</tr>
<tr>
<td></td>
<td>resistance</td>
<td></td>
</tr>
<tr>
<td>amp</td>
<td>Ap^r ampicillin</td>
<td>+12.5 ug/ml chloramphenicol for scoring of unselected markers in LB agar</td>
</tr>
<tr>
<td></td>
<td>resistance</td>
<td>+3 or 10 ug/ml chloramphenicol for selection in R agar</td>
</tr>
<tr>
<td>cat</td>
<td>Cm^r chloramphenicol</td>
<td>+12.5 ug/ml chloramphenicol for scoring of unselected markers in LB agar</td>
</tr>
<tr>
<td></td>
<td>resistance</td>
<td>+3 or 10 ug/ml chloramphenicol for selection in R agar</td>
</tr>
<tr>
<td>tet</td>
<td>Tc^r tetracycline</td>
<td>+25 ug/ml tetracycline for scoring of unselected markers in LB agar</td>
</tr>
<tr>
<td></td>
<td>resistance</td>
<td></td>
</tr>
<tr>
<td>ilv</td>
<td>Ilv^+ isoleucine</td>
<td>- isoleucine and leucine from CDS agar; valine reduced to 20 ug/ml; +1% sodium pyruvate (Brown and Pattee, 1980)</td>
</tr>
<tr>
<td>thr</td>
<td>Thr^+ threonine</td>
<td>- threonine from CDS agar</td>
</tr>
<tr>
<td>thy</td>
<td>Thy^+ purine</td>
<td>- thymine from CDS agar</td>
</tr>
<tr>
<td>trp</td>
<td>Trp^+ uracil</td>
<td>- tryptophan from CDS agar</td>
</tr>
</tbody>
</table>
sterilized in an autoclave, before being stored at 4 C for periods of up to 4 months.

*S. aureus* protoplasts were formed in sucrose-magnesium-tris buffer (SMTB) [0.8 M sucrose, 40 mM MgSO₄, and 100 mM Tris, at pH 7.6]. The MgSO₄ was added first, and allowed to dissolve before adding the remaining reagents. Tris-EDTA-NaCl buffer contained 0.1 M Tris, 0.15 M NaCl, and 0.1 M EDTA at pH 7.5. DNase-free RNase (Sigma) was dissolved in 0.15 M NaCl, pH 5.0. After boiling for 10 min, the pH was adjusted to 7.0, and 1.0-ml aliquots were stored at -10 C. Protease type XIV (Sigma) was dissolved at 10 mg/ml in deionized water, and allowed to self-digest at 35 C for 1 h prior to use. The SLS-ethanol solution contained 5 g of sodium lauryl sulfate (SLS; Nutritional Biochemicals Corporation, Cleveland, OH) in 50 ml of 95% ethanol plus 50 ml of dH₂O. Standard saline citrate (SSC) for dissolving DNA consisted of 6.1 g NaCl, 4.4 g trisodium citrate, and 100 ml deionized water at pH 7.2. A modified SSC solution [designated SSC(2)] was required for DNA hybridization studies. SSC(2) consisted of 8.76 g (0.15 M) NaCl, 4.4 g (0.015 M) sodium citrate, and 1000 ml deionized water at pH 7.0. For denaturing proteins when extracting chromosomal DNA, phenol was distilled under N₂, and was saturated with 0.01 M Tris on the day of use.

The Tris-maleate buffer, used for competent-cell transformation of *S. aureus*, consisted of 0.1 M mono-Tris(hydroxymethyl)aminoethane maleate (Tris-maleate; Sigma) in deionized water at pH 7.0.

Protoplast plasmid transformations were performed in SMMP, which consisted of 2X SMM combined with an equal volume of 4X Penassay broth. The SMM solution was composed of 0.5 M sucrose, 0.02 M maleic acid. (Eastman
Kodak Co., Rochester, NY), and 0.02 M MgCl2, all at pH 6.5 in dH2O.

Polyethylene glycol (PEG) 40% (w/v) of molecular weight 6,000 was used in protoplast plasmid transformation experiments; the PEG solution was filter-sterilized, placed in 10-ml aliquots in 18 x 150-mm screw-capped test tubes, and stored at -70 C. An agar overlay (4 ml) was used to apply the selective agent to either DM3 medium or R agar for some experiments.

The agar overlay, as modified from Novick (1963), consisted of 0.5 M sodium succinate, 0.02 M MgCl2, 0.08% BSA, 0.05 M beta-glycerol phosphate, 1.0% tryptone, 1.0% yeast extract, 0.5% NaCl, 0.5% glucose, 0.1% casamino acids, 0.012% MgSO4, 20 ul trace metals, and 4 g agar per liter. The BSA was sterilized by filtration, and the beta-glycerol phosphate was sterilized in an autoclave; both were added separately to the other already sterilized components. The trace metals solution contained 0.5% (w/v) CuSO4, 0.5% (w/v) ZnSO4, 0.5% (w/v) FeSO4, 0.2% (w/v) MnCl2, in 10% (v/v) HCl.

For extracting plasmid DNA, the SLS-NaOH solution utilized to effect cell lysis consisted of 1% SLS in 0.2 N NaOH. The neutralizing solution of potassium acetate (KAc; 3 M potassium-5 M acetate) was prepared as follows: 60 ml of 5 M KAc (Mallinckrodt Chemical Works, St. Louis, MO), 11.5 ml of glacial acetic acid, and 28.5 ml of deionized water were mixed, and the pH was adjusted to 4.8. Both of the above solutions were prepared fresh on the day of use; the SLS-NaOH solution was prepared from stock solutions of 20% SLS (in deionized water) and 10 N NaOH. For the rapid preparation methods for isolating plasmid DNA, phenol was prepared and stored as described in Maniatis et al. (1982): phenol was distilled under N2, and 8-Hydroxyquinoline (Fisher Scientific Co.) was added at a concentration of
0.1%. The mixture was extracted several times with equal volumes of 1.0 M Tris (pH 8.0), followed by 0.1 M Tris (pH 8.0) plus 0.2% beta-mercaptoethanol (Sigma). The phenol was stored for periods of up to one month at 4 C saturated in equilibration buffer.

Restriction endonucleases were purchased from Bethesda Research Laboratories (BRL; Gaithersburg, MD). A molecular weight standard for estimating the concentration and size of DNA fragments following agarose gel electrophoresis, HindIII-cut lambda DNA, was purchased from New England Biolabs, Beverly, MA. T4 deoxyribonucleic acid ligase (T4 DNA ligase), calf intestinal alkaline phosphatase (CIP), and dithiothreitol (DTT) were purchased from Sigma. Adenosine 5'-triphosphate [dipotassium salt] (ATP) and spermidine, generous gifts from Dr. Robert A. Andrews, were also purchased from Sigma.

Plasmid DNAs were dissolved, diluted, and stored in TE buffer [10 mM Tris, 1 mM EDTA, pH 8.0]. Aliquots of 1-ml and/or 0.1-ml were placed in 1.5 ml Eppendorf polypropylene micro test tubes (Eppendorf tubes; Brinkman Instruments Co., Westbury, NY), and held at either -70 C for long-term storage (periods exceeding three months), or at 4 C for short-term storage. For purification and/or concentration, plasmid DNAs were precipitated as follows: the volume of DNA was estimated, and the concentration of monovalent cations was adjusted by the addition of sufficient 3 M sodium acetate to attain a final concentration of about 0.25 M. To provide a matrix to aid in the precipitation of DNA, 1 ul of a tRNA solution (Sigma; 10 mg/ml in dH2O) was added per ml of DNA dissolved in TE buffer. For small volumes (less than 1.5 ml), it was most convenient to perform the
entire procedure in a single Eppendorf tube. For larger volumes, the precipitation was performed in 50-ml centrifuge tubes (Nalge Company, Rochester, NY). After the addition of a 2X volume of cold (-20 C) 95% ethanol, the solution was mixed thoroughly, and placed at -20 C for 45 min, followed by an additional 15 min incubation at -70 C. Alternatively, the solution was held at -20 C overnight. The DNA was harvested by centrifugation for at least 30 min at 4 C in either a Micro-Centaur micro centrifuge (maximum speed = 11,600 x g; MSE Scientific Instruments, Suffex, England), or a Beckman Model J2-21 centrifuge (10,000 x g; Beckman Instruments, Inc., Palo Alto, CA). The resulting pellet was washed once in 1 to 5 ml of cold (4 C) 70% ethanol, and the residual ethanol was removed by evaporation on a Savant Speed Vac Concentrator (Model SVC-100H, Savant Instruments Inc., Hicksville, L.I., NY). The DNA was resuspended in a minimal (about one-tenth original volume) amount of TE buffer, held at 35 C for about 10 min to assist in dissolving the pellet, and stored as described above.

Plasmid and phage lambda DNAs were analyzed via agarose-gel electrophoresis by using either a horizontal-bed Wide Mini-Sub Cell (mini-gel, 15 x 10 cm tray), or a horizontal-bed DNA Sub-Cell (medium gel, 15 x 20 cm tray; or long gel, 15 x 25 cm tray) electrophoresis system with 1.5 mm-wide 10, 15, or 20 well combs, or a 2-well prep comb. All electrophoresis equipment was purchased from Bio-Rad Laboratories, Richmond, CA. DNA samples (5 to 500 ul) were applied to gels of 0.7 to 1.2% (w/v) Seakem HGT agarose (FMC Corporation, Rockland, ME) or Bio-Rad Ultra Pure DNA Grade agarose (Bio-Rad Laboratories), and fractionated by
electrophoresis in Tris-borate electrophoresis running buffer (TBERB: 89 mM Tris, 89 mM Boric acid, 2.5 mM EDTA) at 3 to 8 volts per cm (constant voltage). To facilitate the loading and to approximate the migration of DNA samples for electrophoresis, a 0.25 volume of tracking dye (0.1% Brom-phenol-blue in 33% glycerol, and 66% TBERB) was added to each sample. After electrophoresis, the gels were stained for approximately 25 min in TBERB containing ethidium bromide (EtBr; Calbiochem-Behring Corp., La Jolla, CA; 1 ug/ml in dH2O). The DNA bands were visualized on a short-wave UV transilluminator (Model LJS-671-500FG, La Jolla Scientific Co. Inc., La Jolla, CA), and photographed with a Polaroid MP-4 Land Camera (Polaroid Corporation, Cambridge, MA), using either Polaroid type 55 P/N or type 57 film and a Wratten #29 gelatin filter (Eastman Kodak Co.). Some gels were also photographed by using 35 mm Plus-X Pan Film (Eastman Kodak Co.) and a Pentax K1000 SE camera (Asahi Optical Co., Japan) fitted with a Wratten #29 gelatin filter.

The customized molecular cloning buffers described below were prepared, with minor modifications, as described in Maniatis et al. (1982). All buffers were prepared at 10X concentration in deionized water, and stored in aliquots of 100 and/or 1000 ul at -20 C for long-term storage (periods exceeding one month), or at 4 C for short-term storage. For digesting DNA with restriction endonucleases, the following restriction endonuclease buffers (REB) were prepared: high-salt REB solution (10 mM DTT, 60 mM MgCl2, 600 mM NaCl, and 1 M Tris-HCl, all at pH 7.5), and medium-salt REB solution (10 mM DTT, 60 mM MgCl2, 600 mM NaCl, and 60 mM Tris-HCl, pH 7.4). In later experiments, DNAs were digested in REB buffer
provided by the supplier of the restriction enzyme (BRL). Two buffers, CIP and STE, were required for dephosphorylating (with calf intestinal alkaline phosphatase) the 5' terminal phosphates from restriction endonuclease digested vector DNA to preclude intra-molecular reannealing. Ammonium sulfate was removed from CIP by centrifugation (11,000 x g, 1 min, 4 C): CIP was redissolved in sterile dH2O and stored at 4 C for up to one month. The CIP buffer used in this study contained 1 mM ZnCl2, 10 mM MgCl2, 10 mM spermidine, and 500 mM Tris-HCl, with the pH adjusted to 9.0. The ZnCl2 and spermidine were prepared fresh at concentrations of 10 mM and 100 mM, respectively, and were diluted for use by addition to the other CIP-buffer reagents. The formulation for STE buffer was 0.01 M EDTA, 0.1 M Tris-HCl and 1 M NaCl, pH 8.0. The ligation buffer used in this study consisted of 7.5 ul of ATP (1.5 mM), 7.5 ul DTT (50 mM), 11.5 ul of sterile dH2O, and 22.5 ul of a solution containing Tris-HCl (0.23 M), MgCl2(30 mM), and BSA (500 ug/ml; filter sterilized).

Plasmid DNA Extraction

Extraction of E. coli plasmid DNA

Plasmid DNAs were extracted from E. coli by using a procedure modified from Maniatis et al. (1982). A 100-ml volume of LB medium in a 300-ml flask was inoculated with a loopful of cells from a fresh 18 x 150 mm BHI agar slant culture of an E. coli strain containing pJH101, or a derivative of pJH101. After overnight incubation in the presence of 30 ug of ampicillin per ml at 35 C with shaking (110 cycles/min, 3.8-cm strokes), each culture was harvested by centrifugation (10,000 x g, 30 min, 4 C), and
washed once with 1X STE (20 ml). The resulting pellet was resuspended in 
lysis buffer (10 ml). At this point, it was often more convenient to store 
the cells in lysis buffer at -20 C (in a sterile 18 x 150 mm screw-capped 
test tube), and continue with the protocol at a later time. If frozen, the 
cells were thawed at room temperature, lysozyme (4 mg/ml) was added, and 
the mixture was held at room temperature for 10 min. Following transfer to 
a 50-ml beaker containing a 1/2-inch magnetic stir-bar, a 20-ml volume of 
SLS-NaOH solution was added gradually over a period of about 2 min with 
gentle stirring, and the mixture was allowed to incubate for 10 min on ice. 
While stirring, cold (4 C) KAc solution (15 ml) was added, and the mixture 
was placed on ice for an additional 10 min incubation. This neutralized 
lysis mixture was transferred to one or more 50-ml screw-capped plastic 
centrifuge tubes, and pelleted at 50,000 x g for 45 min at 4 C. The 
supernatant was carefully removed and placed into 50-ml screw-capped 
plastic centrifuge tubes, to which a 0.6-ml volume of room temperature 
isopropanol was added. The tubes were gently inverted several times, and 
held at room temperature for 15 min before the DNA was harvested (10,000 x 
g, 45 min, room temperature). The pellet was washed once with 5 ml cold 
(-20 C) 70% ethanol and dried under vacuum (about 10 min). After the 
addition of 5 ml TE buffer, and a 15 min incubation at 35 C to assist in 
dissolving the pellet, the plasmid DNA was further purified by CsCl 
gradient centrifugation as described below.

Extraction of S. aureus plasmid DNA

Plasmid DNA was extracted from protoplasts of S. aureus by a 
modification of the Birnboim and Doly (1979) lysis by alkali procedure. A
100-ml volume of GL broth, plus appropriate antibiotics, was inoculated with cells from the strain of *S. aureus* from which DNA was to be extracted. The cells were incubated overnight with shaking as described above for an *E. coli* DNA-extraction starter-culture. For plasmid pI258, and other temperature-sensitive plasmids, this starter culture was incubated at 30°C overnight to preclude loss of the plasmid. The cells were harvested, washed once in 10 ml of sterile saline (3% NaCl), and the resulting pellet was resuspended in 10 ml SMTB. Following transfer to a 50-ml screw-capped Erlenmeyer flask, protoplasts were prepared as follows: a 0.45-ml volume of lysostaphin (1 mg/ml) was added, and the flask was extracted on a Kraft RD-30-18 rotor (Kraft Apparatus Inc., Mineola, NY) for 35 min at 35°C. The contents of the flask were harvested (2,000 x g, 10 min, room temperature) and the supernatant was carefully decanted, and then the protoplasts were gently dislodged from the walls of the tube with the aid of a pipette while immersed in lysis buffer (10 ml). After transfer to a 50-ml screw-capped Erlenmeyer flask, a 1-ml volume of protease solution (10 mg/ml) was introduced, and the flask was roller extracted for 35 min at 35°C. Following transfer to a 50-ml beaker with a 1/2-inch magnetic stir-bar, the SLS-NaOH and KAc solutions were added as described above. The mixture was harvested (50,000 x g, 45 min, 4°C), and the supernatant was carefully decanted in two equal portions into 50-ml plastic centrifuge tubes. A 0.6-ml volume of room temperature isopropanol was added to each tube, and the tubes were gently inverted several times and held at room temperature for 15 min. After harvesting the DNA by centrifugation (10,000 x g, 45 min, room temperature), the pellets were washed once in 5-ml cold (-20°C)
70% ethanol. The supernatant was discarded, and the pellets were dried under vacuum to remove residual ethanol. The DNA was dissolved in 5 ml sterile TE, and then further purified by CsCl gradient centrifugation (see below).

**Rapid preparation of plasmid DNA from E. coli and/or S. aureus**

The following procedure, modified from Birnboim and Doly (1979) and described in Maniatis et al. (1982), was utilized for the detection and preliminary characterization of plasmid DNA from *E. coli* and/or *S. aureus*. It should be noted that this protocol does not yield a highly concentrated nor highly purified preparation of plasmid DNA; however, the DNA obtained can be analyzed by restriction-enzyme digestion. A loopful of cells from a fresh culture of each strain to be analyzed was used to inoculate 5 ml of LB broth (for *E. coli*) or 10 ml of BHI (for *S. aureus*) in an 18 x 150 mm screw-capped test tube. Following overnight incubation at the appropriate temperature in the presence of the desired selective agent(s), a 1.5-ml sample of each strain was transferred into an Eppendorf tube and the cells were sedimented (11,600 x g, 3 min, room temperature). The resulting pellet was resuspended in 100 ul of cold (4 C) lysis buffer containing either 4 mg/ml of lysozyme (*E. coli*), or 40 ul of lysostaphin solution (*S. aureus*). Following 60 min of incubation at room temperature to effect cell wall removal, 200 ul of the SLS-NaOH solution was added; the Eppendorf tube was inverted several times and then was placed on ice for 10 min. A 150 ul volume of cold (4 C) KAc solution was added, and the solution was agitated briefly on a vortex mixer, and returned to ice for an additional 10-min incubation. Cellular debris and chromosomal DNA were sedimented by
centrifugation (11,600 x g, 10 min, 4 C), and the plasmid-containing cleared-lysate (about 425 ul) was transferred to a sterile Eppendorf tube. A 0.5-ml volume of a 1:1 mixture of phenol and chloroform-isoamyl alcohol (24:1 mixture of chloroform and isoamyl alcohol) was added. After gentle mixing, the phases were separated by centrifugation (11,600 x g, 3 min, room temperature). The upper, plasmid-containing phase was transferred to a sterile Eppendorf tube. The DNA was precipitated with ethanol as described previously. The final pellet was dissolved in about 32 ul of TE and stored at 4 C until analyzed.

Chromosomal DNA extraction from S. aureus protoplasts

The protocol outlined below is a modification (Luchansky and Pattee, 1984) of the procedure described by Stahl and Pattee (1983b) for isolating DNA of high molecular weight from protoplasts of S. aureus. A plate (containing 3 ug/ml each of gentamicin and erythromycin and 1 ug/ml of chloramphenicol) was streaked with a strain harboring pPQ126 and incubated at 30 C overnight. The growth was resuspended in 5 ml of sterile saline. Serial 5-fold dilutions of this suspension were made in saline, and 0.1 or 0.25 ml aliquots of these dilutions were used to inoculate duplicate 150-ml volumes of BHI (containing the above concentrations of antibiotics) in 300-ml flasks. The flasks were incubated with gentle shaking at 42 C for about 16 h, and the cells were harvested (10,000 x g, 25 min, 4 C) from flasks of the two highest dilutions exhibiting confluent growth; each pellet was washed once in 10 ml of saline (3.0%). The resulting pellets were resuspended in 5 ml SMTB, duplicate cell suspensions were combined, and protoplasts were prepared as described above (see extraction of S.
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*Staphylococcus aureus* plasmid DNA). A 5-ml volume of Tris-EDTA-NaCl was added, and the pellet was gently dislodged from the wall of the tube with a pipette. The protoplast mixture was transferred to a 50-ml screw-capped Erlenmeyer flask. Following the addition of 0.5 ml of DNase-free RNase, the suspension was incubated at 35 C without shaking for 60 min. Protease (1 ml) was added, and the flask was placed at 35 C for an additional 60 min without shaking. The flask received 0.6 ml SLS-ethanol, and the mixture was shaken vigorously by hand for 1.5 min. Following the introduction of 6 ml of phenol, the contents of the flask were swirled gently, and the mixture was roller extracted for 5 min at room temperature. The phases were separated by centrifugation (10,000 x g, 45 min, 4 C), and the upper DNA-containing phase was transferred to a 50-ml screw-capped Erlenmeyer flask. The DNA was precipitated by the addition of two volumes of cold (-20 C) 95% ethanol, plus sufficient 3 M sodium acetate to attain a final concentration of 0.25 M. The DNA was then spooled onto a glass rod, placed in fresh cold (4 C) 95% ethanol, and held at 4 C overnight. The DNA was aseptically transferred to a test tube containing 3.5 ml of sterile SSC, and stored at 4 C for at least 72 h prior to use.

**CsCl Equilibrium Density Centrifugation**

Plasmid DNA was separated from contaminating chromosomal DNA and cellular debris by cesium chloride (CsCl) equilibrium density centrifugation, essentially as described in Maniatis et al. (1982). 4.5 grams of solid CsCl (Boehringer Mannheim GmbH, West Germany) and 425 ul of ethidium bromide 10 mg/ml in H2O were added to 4.5 ml of a plasmid-containing lysate. The mixture was placed in polyallomer...
quick-seal centrifuge tubes (13 x 51 mm; Beckman Instruments, Inc.) in a VTi65 rotor. After centrifugation at 45,000 rpm for 24 h at 25 °C in a Beckman Model L8-70 (Beckman Instruments Inc.) ultracentrifuge, the maximum density of the cesium gradient was about 1.55 g/ml. Following centrifugation, DNA bands were visualized under short-wave UV light, and removed by using a syringe fitted with an 18-gauge needle. EtBr was removed from the DNA by several extractions with either room temperature isopropanol saturated with 5 M NaCl or a room temperature mixture (1:1) of 1-butanol and water. CsCl was removed by dialysis against four 2-liter changes of TE buffer over 30 h at 4 °C. Plasmid DNA was concentrated by ethanol precipitation. The resulting pellet was resuspended in sterile TE buffer and stored as described previously.

Molecular Cloning

Restriction endonuclease digestion of DNA

DNAs were digested with restriction endonucleases essentially as described in Maniatis et al. (1982): a sample of DNA contained in 20 to 30 μl of REB buffer was exposed to 5 to 10 units (U) of restriction endonuclease per μg of DNA to be digested. REB buffers were used at a sufficient volume to produce a final concentration of 1X. Reaction mixtures were incubated at 37 °C for approximately 1.5 h, before more (one-half dose) enzyme was added. Following an additional 1.5 h incubation at 37 °C, the digestion was stopped by placing the reaction mixture at 67 °C for about 9 min. The digested DNA samples were either loaded directly onto agarose gels or stored at 4 °C until utilized. If stored at 4 °C, the DNA
was incubated at 67 C for 10 min prior to analysis by agarose-gel electrophoresis.

**Dephosphorylation of vector DNA with CIP**

The terminal 5' phosphates on the ends of restriction endonuclease-digested vector DNA (e.g., pJH101 or pTVlts) were removed by treatment with calf intestinal phosphatase (CIP). The procedure was performed, with slight modification, as described in Maniatis et al. (1982). Between 1 and 25 ug of digested vector DNA was precipitated with ethanol. The resulting pellet was resuspended in a nominal volume (10 to 30 ul) of 10 mM Tris-HCl (pH 8.0); 5 ul of 10X CIP buffer and sufficient sterile dH2O were added to bring the total volume to 50 ul. Approximately 0.01 units of CIP were then added per pmole of 5' ends of vector DNA, and the reaction mixture was incubated at 37 C for 30 min. A second aliquot of CIP was introduced, and the mixture was returned to 37 C for an additional 30 min incubation. After adding 40 ul of sterile dH2O, 10 ul of 10X STE, and 5 ul of 10% SLS, the reaction was terminated by placing the mixture at 68 C for 15 min. The CIP-treated DNA was precipitated with ethanol, resuspended in TE buffer, and stored as previously described.

**Isolation of a restriction fragment from agarose by electrophorosis**

Plasmid DNAs were digested with restriction endonuclease(s) as described above, and loaded onto a 1.0% agarose gel fitted with the prep comb. Following exposure to 80 volts constant current for 2.25 h, the gel was stained (see above), and the bands visualized under short-wave UV light. The fragment(s) of interest was excised from the gel with a
razor-blade and forceps. The gel slice was transferred into Spectraphor
dialysis tubing (MW cutoff 12,000 to 14,000; Spectrum Medical Industries,
Inc., Los Angeles, CA) containing a nominal volume of 0.1X TBERB, and the
DNA was eluted from the agarose gel slice by applying a current of 250
volts for 1.5 h. The polarity of the current was reversed for
approximately 30 sec, and the fluid within the tubing was carefully
transferred into a sterile Eppendorf tube containing siliconized glass
wool. A small hole was made in the bottom of the tube, and the entire tube
was placed inside a 17 x 100 mm Falcon tube (Falcon, Oxnard, CA). Residual
chunks of agarose were removed by forcing the solution through the glass
wool via centrifugation (2,000 x g, 4 min, room temperature). The eluate
collected in the Falcon tube was transferred to a sterile Eppendorf tube,
and the DNA was precipitated and stored as described above.

Ligation of plasmid DNAs with T₄ DNA ligase

The ligation procedure utilized in this study was modified from a
procedure described by Macrina et al. (1980). A 50-ul volume of
restriction endonuclease-digested, CIP-treated vector DNA plus restriction
endonuclease-digested passenger DNA was mixed with 50 ul of ligation
buffer. The total concentration of DNA was approximately 1 ug, and the
ratio of complementary ends of passenger DNA to ends of vector DNA was
about 50 to 1. T₄ DNA ligase (100 U/ml) was added, and the mixture was
held at 15 C for 15 h. The DNA was precipitated with ethanol. The pellet
was resuspended in SSC-CaCl₂ (1X SSC plus 0.1 M CaCl₂) or TE buffer, and
used directly for transformation of either E. coli or S. aureus,
respectively.
Transfer of DNA from agarose to nylon membranes and detection of specific sequences via DNA hybridization

Chromosomal and plasmid DNAs were purified by CsCl-gradient centrifugation, digested with a restriction endonuclease(s), and fractionated by agarose-gel electrophoresis. Lambda DNA was digested with HindIII or PstI and included on all gels as a molecular weight standard and as a negative control for hybridizations. After the DNA was stained and photographed, the gel was incubated with gentle shaking at room temperature while submerged successively in depurination (0.25 M HCl), denaturation (0.5 M NaOH, 1.5 M NaCl), and neutralization (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl) solutions for 8 min, 45 min, and 90 min, respectively. A sheet of Magnagraph (biochemically optimized nylon solid support membrane; Micron Separations Inc., Honeoye Falls, NY) was cut to the exact size of the gel, and both the membrane and gel were allowed to equilibrate for about 5 min in Tris-acetate buffer [(TAE); 0.04 M Tris-acetate, 0.002 M EDTA, pH 8.0]. The DNA was then transferred from agarose to Magnagraph using a Hoefer Scientific Instruments (HSI; San Francisco, CA) electroblot apparatus. Briefly, the membrane and gel were juxtaposed within a specialized cassette and placed into the HSI Transphor (TE 42) tank. Fragments of DNA were transferred from agarose to Magnagraph using TAE buffer (room temperature) at 400 milliamps (less than 10 volts) for 150 min. Upon completion of the transfer, the membrane was rinsed briefly in 2X SSC(2) and baked under vacuum at 80 C for 2 to 4 h. The baked membrane was stored at 4 C in a Micro-Seal sealable pouch (model 6011; Dazey Corporation, Industrial Airport, KS) until utilized.
DNA was labeled with alpha $^{32}\text{P}$-dCTP by nick translation using a nick translation kit purchased from BRL. Labeled DNA was separated from unincorporated nucleotides by ethanol precipitation. Ethanol precipitation was performed as described previously, except one-half volume of 7.5 M ammonium acetate was substituted for sodium acetate. The labeled DNA was denatured by boiling for 10 min and added to the hybridization solution at a specific activity of about $10^7$ cpm/ug with a final probe concentration of 25 to 50 ng/ml.

The following solution (excluding probe) was used for both prehybridization and hybridization at 100 ul/cm$^2$ membrane and 50 ul/cm$^2$ membrane, respectively: 6X SSC(2), 50% deionized formamide, 0.2% bovine serum albumin, 0.2% ficoll (molecular weight 400,000), 0.2% polyvinylpyrrolidine, 1.0% SLS, and 50 ug/ml of denatured salmon sperm DNA. The prehybridization solution was added to the Micro-Seal pouch and incubated at 42 C with constant agitation for at least 8 h. After prehybridization, the solution within the pouch was discarded, and hybridization solution and denatured radioactive probe were added and the mixture was incubated at 42 C with constant agitation for an additional 16 to 30 h. Following hybridization, the solution was discarded and the membrane was washed successively in three different solutions to remove unbound and nonspecifically bound radioactive probe using an Omniblot processing system (American Bionetics Inc., Emeryville, CA). The wash solutions were: 10X SSC(2), 0.01% SLS (wash 1); 1X SSC(2), 1% SLS (wash 2); and 0.1X SSC(2), 0.1% SLS (wash 3). Wash solution 2 was used at 37 C, and wash solutions 1 and 3 were utilized at room temperature. The Omniblot system was operated
with a flowrate of about 50 ml/min, and each wash cycle was 5 to 10 min in length (total volume of 250 to 500 ml for each wash solution). Following the wash regimen, the membrane was removed from the Omniblot bag and wrapped in plastic. DNA fragments that hybridized to the probe were visualized by autoradiography following exposure to Kodak X-Omat X-Ray film for 1 to 3 days. The film cassette was placed at -70°C because a Cronex intensifying screen (Dupont) was used to enhance the intensity of the autoradiogram and/or to minimize the time required for exposure. The X-Ray film was developed in Kodak GBX developer.

Competent-Cell Transformations

_E. coli_ competent-cell transformation

A modification of the procedure of Dagert and Ehrlich (1979) was used to transform _E. coli_ with plasmid DNAs. The cells from an overnight BHI agar slant (18 x 150 mm) culture of the _E. coli_ strain to be transformed were suspended in sterile saline; 2 ml of the cell suspension were used to inoculate a 100 ml-volume of LB broth. This flask was incubated with shaking at 35°C until an optical density at 540 nm [O.D. (540)] of 0.45 was attained. After being held on ice for 25 min, the cells were harvested (10,000 x g, 30 min, 4°C), and the resulting pellet was resuspended in 50 ml of sterile 0.1 M MgCl₂. The cells were sedimented again (10,000 x g, 15 min, 4°C). The pellet was resuspended in 5 ml of sterile 0.1 M CaCl₂ and held at 4°C for between 16 and 24 h. A 200-ul volume of competent cells was added to an Eppendorf tube containing either 100 ul (about 50 to 150 ng) of plasmid DNA dissolved in SSC-CaCl₂ (experimental), or 100 ul
SSC-CaCl₂ (cell control tube). Following a brief heat-shock treatment (43 C for 2 min), the tubes were returned to ice for 30 min. The contents of each tube were diluted to 1 ml with LB broth and placed at 37 C for 60 min. 0.1-ml aliquots (with prior dilution) were plated onto selective media. Colony-forming units (cfu) were assayed by spreading diluted samples of the control tube onto duplicate LB agar plates. The reversion frequency was determined by plating 0.1-ml aliquots of the undiluted cell control onto selective media. All plates were incubated at 35 C for about 25 h. Colonies were counted, and plates with transformants were replicated onto appropriate media to score unselected markers. After incubation of the replica plates for about 30 h at 35 C, they were scored.

**S. aureus competent-cell transformation**

Recipient cells for competent-cell transformation of *S. aureus* were prepared essentially as described by Pattee and Neveln (1975) and Stahl and Pattee (1983b). Cells from an overnight BHI agar slant (18 x 150 mm) culture of the recipient strain were suspended in sterile saline (0.85%), and used to inoculate a 100-ml volume of Oxoid TSB to an O.D.(540) of 0.1. Ten ml of this suspension were added to each of six 300-ml nephelometer flasks, each containing 100 ml of Oxoid TSB plus 1 mM CaCl₂. The flasks were incubated with gentle shaking at 35 C until an O.D.(540) of 0.05 was attained. After the addition of 1 ml of normal rabbit serum (Pel-Freez Biologicals, Rogers, AZ) to each flask, the cells were returned to 35 C until an O.D.(540) of 0.1 was reached. The cells from the six flasks were harvested (10,000 x g, 25 min, 4 C), and the resulting pellets were combined into 1.5 ml of TSB. TSB (0.5 ml), normal rabbit serum (1 ml), and
phage 55 (0.25 ml; titer = 1 to 2 x 10^{11} pfu/ml) were added to the pooled cells. Following a 5-min incubation at 35 C with gentle shaking, the cells were harvested by centrifugation and washed in 9 ml of cold Tris-maleate buffer. The washed cells were resuspended in 6 ml of cold Tris-maleate buffer containing 0.1 M CaCl_2, and 1-ml aliquots were added to tubes containing either 0.15 ml of transforming DNA (experimental) or 0.15 ml of SSC (control). The tubes were mixed gently and were held for 3 min at 4 C and then 3 min at 35 C with gentle shaking. The cells were harvested by centrifugation. The resulting pellets were resuspended in 3 ml of BHI containing 2 mM sodium citrate; the mixture was incubated for 1 h at 35 C with gentle shaking. When appropriate, 0.1 ug/ml of either erythromycin or chloramphenicol was added to a mixture to induce certain genes (e.g., erm of Tn917, or cat of pC194). The cells were harvested by centrifugation. Each pellet was resuspended in 1 ml of sterile saline (0.85%). 0.1-ml aliquots of the control and experimental suspensions (without prior dilution) were spread onto the appropriate selective media. Diluted samples (0.1 ml) of the control suspension were also spread in duplicate onto BHI agar plates to determine total cfu. After incubation of the plates at 35 C for approximately 40 h, colonies were counted and the transformants were replica-plated onto appropriate media to score for unselected markers. Replica plates were scored following 30 h of incubation at 35 C.

Protoplast Plasmid Transformation

The protoplast plasmid transformation protocol for S. aureus described below is a modification of the Chang and Cohen (1979) procedure for
transforming protoplasts of *B. subtilis* with plasmid DNA. Cells of a recipient strain, grown overnight on an 18 x 150 mm BHI agar slant culture at 35°C, were uniformly suspended in 5 ml of sterile saline. A sufficient volume of this suspension was used to inoculate a 300-ml nephelometer flask containing 100 ml of Penassay broth to an O.D. (540) of 0.04. The flask was incubated at 35°C with shaking until an O.D. (540) of 0.62 was attained. The cells were harvested by centrifugation (10,000 x g, 25 min, 4°C), and the pellet was washed once with 10 ml of 3% NaCl. A 10-ml volume of SMTB was added to resuspend the resulting pellet, and the suspension was transferred to a 50-ml screw-capped Erlenmeyer flask. Following the addition of 0.45 ml of lysostaphin solution, the mixture was extracted on a roller apparatus for 45 min at 35°C. The mixture was transferred to a 50-ml plastic centrifuge tube, and the protoplasts were pelleted by centrifugation (2,000 x g, 15 min, 4°C). SMMP (5 ml) was added, and the pellets were gently dislodged from the walls of the tube with the aid of a pipette. The protoplasts were again sedimented by centrifugation (1,000 x g, 6 min, 4°C). The pellet was resuspended in 2 ml of SMMP. An aliquot of the protoplast suspension was diluted in SMMP. Various dilutions were plated in duplicate onto TSA and DM3 and/or R agar to determine non-protoplasted units and regeneration frequency, respectively.

Plasmid DNA (0.5 to 2 ug, usually in a volume of 100 ul) was mixed with an equal volume of 2X SMM in a sterile 50-ml plastic centrifuge tube (experimental). A control tube, containing 2X SMM and an equal volume of TE buffer, was also prepared. A 0.5-ml volume of the protoplast suspension was added to each tube, usually followed immediately by the addition of 1.5
ml of PEG. In some experiments, the protoplasts were incubated at 56 C for 45 seconds before addition to the DNA/SMM solution. The tubes were slowly inverted for 3 to 4 min, and then 5 ml of SMMP were added to each tube. After inverting the tube 2 or 3 times, the solution was harvested (1,000 x g, 6 min, 4 C). The resulting pellets were carefully removed from the walls of the tube with the aid of a pipette while immersed in SMMP (1 ml). The protoplast-DNA mixture was either plated immediately (plating method 1), or allowed to incubate for 3.5 h at 30 C without shaking before plating (plating method 2). The protoplast-DNA mixture was plated onto DM3 medium or R agar that either directly contained the selective agent, or was supplied with the selective agent via an agar overlay. In some experiments, the protoplast-DNA mixture was incubated in the presence of low levels of the selective agent (0.1 ug/ml) during the 3.5-h incubation at 30 C, to induce enzyme formation. Protoplasts were plated with and without dilution onto selective media, and were incubated at high humidity for 5 to 10 days at 30 C. Selective levels of antibiotics were 3 or 10 ug/ml for chloramphenicol, and 1 or 1.5 ug/ml for erythromycin. The presence of plasmid-containing transformants was verified by their resistance to higher concentrations of antibiotic, and by the subsequent isolation of plasmid DNA via rapid preparation methods.

Transduction

The phage phill lysate used for all transduction experiments was prepared as described previously (Schroeder and Pattee, 1984). Transductions were performed by using the procedure described by Kasatiya and Baldwin (1967), as modified by Schroeder and Pattee (1984). Cells from
an overnight BHI agar slant (18 x 150 mm) culture of the recipient strain were harvested into 1 ml of TSB. Aliquots of 0.5 ml (between 1 and 5 x $10^{10}$ cfu) were transferred to two 15-ml Corex centrifuge tubes. Sufficient transducing lysate (usually 0.25 to 0.5 ml) was added to the experimental tube to obtain a multiplicity of infection (moi) of one. The volumes of the experimental and control tubes were brought to 2 ml with TSB containing 5 mM CaCl$_2$. The contents of the tubes were separately mixed and incubated with vigorous shaking at 30 C for 20 min. After the addition of 1 ml of cold (4 C) 20 mM sodium citrate to each tube, the cells were harvested by centrifugation. Each pellet was resuspended in 1 ml of cold (4 C) 20 mM sodium citrate. Aliquots (0.1 ml) were spread onto the appropriate selective media. Diluted samples (0.1 ml) from the control suspension were spread in duplicate onto BHI agar plates to determine total cfu. All plates were incubated at 30 C for 40 h before colonies were counted, and transductants were replica plated onto appropriate media to score for unselected markers. After incubating for approximately 30 h at 30 C, the replica plates were scored.

**Plasmid Elimination Experiment**

Bacteria carrying a temperature-sensitive plasmid were incubated on agar plates with appropriate selection for about 30 h at 30 C. A 5-ml volume of sterile saline (0.85%) was poured onto the plates, and the cells were removed from the surface of the plates by using a glass spreader (usually 1 to 5 x $10^{10}$ cfu/ml). After brief mixing, serial 10-fold dilutions were made in saline, and the cells were plated onto agar plates with and without antibiotics. Duplicate plates of each dilution were
incubated at 30 C and 39 and/or 43 C. Several isolates from plates incubated at each temperature were analyzed for the presence or absence of plasmid DNA by scoring for unselected plasmid markers, and by the isolation of plasmid DNA with the rapid preparation method.
RESULTS

Preliminary Observations

The aim of this project was to construct and employ an integrable plasmid to facilitate the genomic analysis of S. aureus NCTC 8325. Because several well-characterized B. subtilis insertion vehicles were already available (Ferrari et al., 1983; Haldenwang et al., 1980; Niaudet et al., 1982; Saunders et al., 1984a), attempts were made to adapt an established Gram-positive insertion vector (pJH101) for use in S. aureus. A portion of Tn551 was cloned onto pJH101 (pLUCH3) to extend the utility of this vector: plasmid integration directed into any region of the chromosome containing an insertion of Tn551. For several reasons, most notably the reliance on resistance to chloramphenicol to select for plasmid integration, it was not possible to recover chromosomal insertions of pLUCH3 in S. aureus. The efforts to adapt pLUCH3 for use in S. aureus are summarized below, because many of these early results contributed to the strategy used to construct a novel S. aureus plasmid insertion vector, pFQ126.

Restriction endonuclease characterization of pJH101 and pI258

Before using pJH101 and pI258 in molecular cloning experiments, it was necessary to digest both plasmids with restriction enzymes to verify the presence of certain cleavage sites. Figure 6 contains the EcoRI and HindIII restriction endonuclease analyses of pJH101 and pI258. Lanes 1 and 2 contain pJH101 linearized with EcoRI and HindIII, respectively: the single band in each lane was the expected result for EcoRI or
Figure 6. **EcoRI** and **HindIII** restriction analyses of CsCl-purified plasmids pJH101 and pI258

Lane 1, **EcoRI**-digested pJH101; lane 2, **HindIII**-digested pJH101; lane 3, **HindIII**-digested lambda standard (0.35 ug total); lane 4, **HindIII**-digested pI258; lane 5, **EcoRI**-digested pI258.
HindIII-digested pJH101 based on the restriction map for pJH101 published by Ferrari et al. (1983). The four distinct bands visible in lane 5 following digestion of plasmid pI258 with EcoRI compare exactly with the EcoRI-digestion pattern for pI258 published by Novick et al. (1979b). The HindIII restriction endonuclease analysis of pI258 (lane 4) shows the presence of 13 distinct bands, and perhaps an additional fragment faintly visible below the 0.6 kb band of HindIII-digested lambda DNA. Wilson and Baldwin (1978) reported a total of 11 fragments following digestion of plasmid pI258 with HindIII. The pattern of the first 11 fragments (HinA to HinK) compares favorably with the HinA through HinK fragments published by Wilson and Baldwin (1978). Two plausible explanations could account for the absence of the HinL, M, and N fragments from the work of Wilson and Baldwin: 1) not enough DNA was loaded onto the gel for these bands to be visualized; and/or 2) plasmid pI258 was not completely digested, and the three smaller bands remained associated with higher molecular weight DNA. Either or both of the above possibilities could account for the discrepancy.

Molecular cloning of the erythromycin-resistance determinant from plasmid pI258 into the HindIII site of pJH101

The *ermB* determinant (of transposon Tn551) was reported to reside on a 2.8-md fragment of HindIII-digested pI258 (Wilson and Baldwin, 1978). One objective of this study was to clone the erythromycin resistance determinant of pI258 onto an insertion vector. The results of two different approaches to accomplish this goal are described below.
The first approach was to "shotgun" clone the erythromycin resistance determinant of pI258 onto the insertion plasmid pJH101. Both pI258 and pJH101 were digested with HindIII; the resulting fragments were ligated and transformed into competent cells of E. coli strain HB101, as described previously. Among the ampicillin-resistant transformants generated in this manner, it seemed probable that the 2.8-md HinB fragment would be present in at least one clone. Several phenotypic (Am^Cm^Tc^) insert-containing clones were obtained (data not shown). Each isolate was streaked for isolation, and a single colony of each was retained for further study. Plasmid DNA was extracted from each isolate by the rapid preparation method, digested with HindIII, and subjected to electrophoretic analysis. Of all the presumptive insert-containing isolates tested (data not shown), only one isolate (JBL6) contained a segment of S. aureus passenger DNA. Analysis of DNA isolated from JBL6 confirmed the presence of a recombinant plasmid, pLUCH4, harboring the HinG fragment of plasmid pI258 (see below). The "shotgun" approach to cloning the HinB fragment of pI258 was not successful.

Next, the 2.8-md fragment of HindIII-digested pI258 was isolated directly from agarose and ligated to pJH101 to increase the probability of obtaining a recombinant plasmid containing the ermB gene from plasmid pI258. The ligation mixture of CIP-treated, HindIII-digested pJH101 plus the HinB fragment of pI258 was used to transform E. coli strain HB101. Transformants were selected for resistance to ampicillin, and then scored for resistance to chloramphenicol and for tetracycline sensitivity. Approximately 7% (7 of 105 total) of the Ap^ transformants recovered were
also Cm\(^\text{R}\) and Tc\(^\text{S}\); however, two of the 7 Am\(^\text{R}\)Cm\(^\text{R}\)Tc\(^\text{S}\) clones were also resistant to 300 ug of erythromycin per ml (data not shown). After selecting a single colony from each Tc\(^\text{S}\) transformant from streak plates, all 7 Am\(^\text{R}\)Cm\(^\text{R}\)Tc\(^\text{S}\) putative insert-containing clones were retained for further study.

To determine if any of the Am\(^\text{R}\)Cm\(^\text{R}\)Tc\(^\text{S}\) isolates contained recombinant molecules, plasmid DNA was extracted from each clone. Following digestion of the CsCl-purified plasmid DNA with HindIII, each of the isolates, along with HindIII-cut pI258 and pJH101, were analyzed by agarose-gel electrophoresis (data not shown). Of the 7 Am\(^\text{R}\)Cm\(^\text{R}\)Tc\(^\text{S}\) clones tested, only the two clones that were also resistant to erythromycin contained pJH101 plus S. aureus passenger DNA. Figure 7 presents evidence that the 2.8 md HindB fragment from plasmid pI258 was cloned into the HindIII site of pJH101. pLUCH2 (lane 9) and pLUCH3 (lane 10) each display 2 distinct bands following digestion with HindIII. The larger fragment migrated to the same position as HindIII-linearized pJH101 (lane 11), and the smaller fragment to the same position as the HindB (ermB determinant) of HindIII-digested pI258 DNA (lane 7). Figure 7 also contains results of the "shotgun" cloning of the HindG fragment of pI258 onto pJH101 (- pLUCH4). By comparing the HindIII-digestion pattern of pLUCH4 (lane 8) to the HindIII patterns for plasmids pI258 and pJH101 (lanes 7 and 11, respectively), pLUCH4 was defined as pJH101 plus the HindG fragment of pI258. Uncut pJH101, pLUCH4, pLUCH3, and pLUCH2 are shown in lanes 2, 3, 4, and 5, respectively. The migration patterns of the uncut plasmids were also consistent with the size of the passenger DNA in pLUCH2, pLUCH3, and pLUCH4. pJH101, the smallest
Figure 7. Electrophoretic analysis of CsCl-purified preparations of uncut and HindIII-digested plasmids pJH101, pLUCH4, pLUCH3, pLUCH2, and pI258

Lane 1, HindIII-digested lambda standard (0.32 ug total); lane 2, uncut pJH101; lane 3, uncut pLUCH4; lane 4, uncut pLUCH3; lane 5, uncut pLUCH2; lane 6, HindIII-digested lambda standard (0.32 ug total); lane 7, HindIII-digested pI258; lane 8, HindIII-digested pLUCH4; lane 9, HindIII-digested pLUCH3; lane 10, HindIII-digested pLUCH2; lane 11, HindIII-digested pJH101; lane 12, HindIII-digested lambda standard (0.32 ug total).
plasmid, migrated farther than the next largest plasmid, pLUCH4, that migrated farther than pLUCH2 and pLUCH3.

From the results presented in Figure 7, two isolates were verified to contain the 2.8-md fragment of plasmid pI258 cloned into the HindIII site of pJH101: JBL4 contained pLUCH2 (lane 10), and JBL7 contained pLUCH3 (lane 9). It is not precisely known why 5 of the 7 presumptive clones (Am^Cm^Tc^S) did not contain inserts of S. aureus passenger DNA. Efforts were directed to determine the precise orientation of the HinB fragment in pLUCH2 and pLUCH3. Ferrari et al. (1983) determined that pJH101 contained a single cleavage site for the restriction endonuclease PstI. Wilson and Baldwin (1978) reported that the HinB fragment from plasmid pI258 contained a single PstI site, positioned very near one end of the fragment. The digestion of pLUCH2 and pLUCH3 with PstI would be expected to yield 2 distinct fragments for each plasmid (one PstI site each in the vector and passenger segments of these plasmids). Furthermore, if the HinB fragment were present in the same orientation in both pLUCH2 and pLUCH3, then the PstI restriction pattern would be identical for these two plasmids. Restriction endonuclease digestion of pLUCH2 and pLUCH3 with PstI generated the expected two fragments from each plasmid: the migration patterns were identical (data not presented). Because pLUCH2 and pLUCH3 both contained the HinB fragment in the same orientation, pLUCH3 was chosen arbitrarily for further study.

Presentation of pLUCH3 to competent cells of S. aureus

Competent cell transformation was the approach initially used in attempts to introduce pLUCH3 into S. aureus. As shown in Table 4,
Table 4. Transformation of strain ISP130 with chromosomal DNA from strain ISP1010 and plasmid DNA from strains ISP479, ISP1213, JBL7, and ISP856

<table>
<thead>
<tr>
<th>Selection for:</th>
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<th>Cm&lt;sup&gt;r&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformation</td>
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<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Reversion</td>
<td>6</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
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Relevant markers

<table>
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<tr>
<th>Donors:</th>
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<th>Cm</th>
</tr>
</thead>
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<tr>
<td>ISP1010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISP479</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISP1213</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JBL7</td>
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</tr>
<tr>
<td>ISP856</td>
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<td></td>
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<td>ISPs:</td>
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<td>ISP1010</td>
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<tr>
<td>ISP1213</td>
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<td></td>
</tr>
<tr>
<td>ISP856</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis of phenotypes of transformants:

ISP1010

- Of 55 Em<sup>r</sup> transformants: 55 (100%)
- Of the Cm<sup>r</sup> transformants: 14

ISP130 8325-4 [pI258 blaT443 asa-33 ermB20 repA18] 500 pig-131
ISP1010 8325 mec-4916 nov-142 trpE85 rib127 tnm3106 uraB232::Tn551 ermB321
ISP479 8325-4 pig-131 (pI258)
ISP1213 E. coli MM294 (pJH101)
JBL7 E. coli HB101 (pLUCH3)
ISP856 8325-4 (pC194).

<sup>b</sup>Expressed for ISP1010 DNA as colonies recovered per 10<sup>9</sup> cfu; 1.88 x 10<sup>9</sup> cfu per ml of the transformation suspension and excess ISP1010 DNA were used.

<sup>c</sup>Lawn of cells present due to basal-level resistance of ISP130 to approximately 4 ug of chloramphenicol per ml.
Table 4 (Continued)

<table>
<thead>
<tr>
<th>Donors:</th>
<th>Relevant markers</th>
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</thead>
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<tr>
<td>pI258</td>
<td>Em</td>
</tr>
<tr>
<td>pJH101</td>
<td>R</td>
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<tr>
<td>pLUCH3</td>
<td>R</td>
</tr>
<tr>
<td>pC194</td>
<td>S</td>
</tr>
</tbody>
</table>

Recipient:

Analysis of phenotypes of transformants:

- **pI258**
  - Of 2 Em<sup>R</sup> transformants
    - 2 (100%)
    - Of the Cm<sup>R</sup> transformants<sup>c</sup>

- **pJH101**
  - Of 3 Em<sup>R</sup> transformants
    - 3 (100%)
    - Of the Cm<sup>R</sup> transformants<sup>c</sup>

- **pLUCH3**
  - Of 1 Em<sup>R</sup> transformant
    - 1 (100%)
    - Of the Cm<sup>R</sup> transformants<sup>c</sup>

- **pC194**
  - Of 2 Em<sup>R</sup> transformants
    - 2 (100%)
    - Of the Cm<sup>R</sup> transformants<sup>c</sup>

<sup>d</sup>Approximately 10 μg of non-CsCl purified plasmid DNA was used.
competent cells of ISP130 were transformed with the following plasmids: pC194, pJH101, pI258, and pLUCH3. When selection was made for resistance to erythromycin by using chromosomal DNA (ISP1010), Em\(^R\) Cm\(^S\) transformants were recovered at a frequency of 197 per 10\(^9\) cfu. The recovery of Em\(^R\) transformants with ISP1010 DNA indicated that ISP130 was indeed competent for DNA uptake. As expected, however, it was not possible to recover any Cm\(^R\) transformants using ISP1010 DNA. It should be noted, that the strains of *S. aureus* used in this study had a basal-level resistance to about 4 ug per ml of chloramphenicol. Although higher selective levels of chloramphenicol lowered the amount of background growth observed, it was assumed that higher levels also reduced the number of Cm\(^R\) transformants; therefore, Cm\(^R\) transformants were selected on media containing 4 ug of chloramphenicol per ml. The background lawn was distinguished from Cm\(^R\) transformants by scoring for resistance to 10 ug per ml of chloramphenicol.

Fourteen Em\(^R\) isolates were obtained with ISP1010 DNA when selection was made for resistance to chloramphenicol. These Em\(^R\) isolates probably represent reversion of the *ermB20* mutation of ISP130. It was also possible that recombination between the two copies of *ermB* (*ermB327* in ISP1010 and *ermB20* in ISP130) produced the Em\(^R\) transformants.

For plasmid pI258, two Em\(^R\) Cm\(^S\) transformants were obtained from erythromycin selection plates, and one Em\(^R\) transformant was recovered from chloramphenicol selection plates. These Em\(^R\) transformants are probably spontaneous revertants of the erythromycin-sensitive mutation (*ermB20*) of Tn551 in ISP130. As shown in Table 4, similar results were also obtained for pJH101, and pLUCH3.
As a positive control for the selection of Cm^r transformants, pC194 was presented to competent cells of ISP130; only seven Cm^r Em^s isolates were recovered. Lofdahl et al. (1978a) reported a frequency of 1000 Cm^r resistant transformants per 1 ug of pC194 DNA introduced to competent cells of S. aureus. One explanation for the low recovery of pC194 transformants, was that the plasmid DNA utilized for this experiment was not purified on CsCl (see below). It is also possible that the higher frequency observed by Lofdahl et al. (1978a), was a consequence of the recipient strain. Because of the high number of cells present, it was not surprising to recover two Em^s Cm^r isolates on the erythromycin selection plates.

The results in Table 4 demonstrated that competent cells of ISP130 were not amenable to transformation with plasmid DNA; plasmid pJH101, isolated from E. coli and lacking a Gram-positive origin of replication, yielded about the same low number of Em^r colonies as the other plasmids tested. Although several additional attempts were made to transform competent cells of various strains of S. aureus with plasmid DNA, these experiments were not successful (data not shown).

Presentation of pLUCH3 to protoplasts of S. aureus

From the results presented in Table 4, and similar data from several additional experiments, it was obvious that competent cells of S. aureus were recalcitrant to transformation by plasmid DNA. As an alternate method for introducing the insertion vector pLUCH3 into S. aureus, attempts were made to transform protoplasts with plasmid DNA. The protocol was performed as described previously, with the following exceptions: plasmid DNAs were not purified by CsCl-gradient centrifugation, and protoplasts were not
heated prior to the addition of plasmid DNA.

Table 5 contains data representative of results obtained for introducing plasmid DNA into *S. aureus* protoplasts. To better compare protoplast to competent-cell transformation, ISP130 was used as the recipient for the protoplast plasmid transformation experiment in Table 5. Because pLUCH3 contained two Gram-positive selectable markers, it was possible to select for erythromycin- or chloramphenicol-resistant transformants. Regardless of the antibiotic used for selection, however, no pLUCH3 transformants of ISP130 were obtained.

Erythromycin-resistant transformants were obtained at low frequency (8.6 x 10^0 per μg of DNA) when protoplasts of ISP130 were transformed with plasmid pI258 DNA. Chloramphenicol-resistant transformants were recovered at a frequency of 1.18 x 10^4 per μg when plasmid pC194 DNA was introduced to protoplasts of ISP130. Plasmid pI258 (28.2 kb) is considerably larger than pC194 (2.9 kb), and the size differential may account in part for the difference in recovery of these plasmids following transformation of ISP130 protoplasts. Upon comparison of the data in Tables 4 and 5 it was apparent that *S. aureus* was more amenable to plasmid uptake via protoplast plasmid transformation than by competent-cell transformation. Nevertheless, neither the protoplast nor the competent-cell transformation procedures were successful for transferring plasmid pLUCH3 from *E. coli* into *S. aureus*. Based on the results of the preliminary experiments, efforts were directed to improve the efficiency of plasmid DNA uptake via transformation of *S. aureus* protoplasts.
Table 5. Transformation of protoplasts of strain ISP130 with plasmid DNA from strains ISP856, ISP479, ISP1213, and JBL7\(^a\)

<table>
<thead>
<tr>
<th>Selection for:</th>
<th>1 ug/ml Em</th>
<th>3 ug/ml Cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>pC194</td>
<td>0</td>
<td>1.18 (\times) 10(^4)</td>
</tr>
<tr>
<td>pI258</td>
<td>8.6 (\times) 10(^0)</td>
<td>0(^c)</td>
</tr>
<tr>
<td>pJH101</td>
<td>0</td>
<td>0(^c)</td>
</tr>
<tr>
<td>pLUCH3</td>
<td>0</td>
<td>0(^c)</td>
</tr>
</tbody>
</table>

\(^a\)ISP130 8325-4 w[pI258 blaA443 asa-33 ermB20 repA18]500 pig-131
ISP856 8325-4 (pC194)
ISP479 8325-4 pig-131 (pI258)
ISP1213 E. coli MM294 (pJH101)
JBL7 E. coli HB101 (pLUCH3).

\(^b\)Expressed as protoplast plasmid transformants recovered per 1 ug of plasmid DNA.

\(^c\)Lawn of cells present due to basal-level resistance of ISP130 to approximately 4 ug of chloramphenicol per ml; the lawn was sensitive to 10 ug per ml of both erythromycin and chloramphenicol.
Protoplast Plasmid Transformation

As described in the previous section, protoplast plasmid transformation was a more efficient method, compared to competent cell transformation, for introducing plasmid DNA into *S. aureus*. Efforts were directed, therefore, to optimize the conditions for protoplast plasmid transformation to increase the frequency of plasmid uptake. The experimental parameters of the protoplast plasmid transformation procedure examined were: conditions for plating; regeneration media used for selection of plasmid-containing transformants; quality of plasmid DNA (CsCl purified vs non-CsCl purified DNA); induction of plasmid-borne inducible resistance determinants; and heat treatment of protoplasts prior to the addition of DNA.

Lindberg vs Stahl and Pattee protocol for preparing and plating protoplasts

The preparation of *S. aureus* protoplasts, plating conditions, and regeneration media, were investigated by using either the Lindberg (1981) and/or the Stahl and Pattee (1983a) methods for handling protoplasts. Several aspects of these procedures are variations of the Chang and Cohen (1979) protocol for transforming *B. subtilis* protoplasts with plasmid DNA. Parameters were compared between both methods to determine the combination resulting in the highest frequency for plasmid transformation.

Several attempts were made to prepare protoplasts of *S. aureus* as described by Lindberg (1981), but these efforts were not successful. Colonies were obtained from control plates for total cfu, non-protoplasted units, and total protoplasts (on both DM3 and R-agar); however, plasmid
transformants were not recovered from the experimental plates when the Lindberg method was used for preparing protoplasts (data not shown). One explanation for the failure to recover transformants was the low efficiency of protoplast formation: of approximately $10^9$ cfu, only about $10^4$ regenerated protoplasts were obtained from control plates (data not shown). The paucity of regenerated protoplasts precluded the recovery of protoplast plasmid transformants. Further attempts to increase the efficiency of protoplast formation with the Lindberg method were not attempted. For this reason, protoplasts were prepared by the method of Stahl and Pattee (1983a) in the experiments that follow.

Table 6 presents results obtained from transforming protoplasts of ISP874 with plasmid pC194 DNA to determine the most efficient plating regimen, as well as the proper medium and selective conditions for recovering protoplast plasmid transformants. A concentration of 10 ug per ml of chloramphenicol was used for selection (compared to 4 ug per ml used in previous experiments) to eliminate the lawn of cells that appeared earlier on control and experimental plates due to the basal level of chloramphenicol resistance. More transformants were obtained when chloramphenicol was incorporated directly into DM3 media, as opposed to an agar overlay (compare columns A and B of Table 6). For R-agar, more transformants were recovered when an agar overlay was used, compared to direct incorporation of the antibiotic (columns C and D). With one exception (compare plating method 2 for column B vs D), R-agar yielded more transformants than DM3 agar, regardless of the conditions for plating or the manner in which the antibiotic was added. Table 6 also compares
Table 6. Transformation of protoplasts of strain ISP874 with plasmid DNA from ISP856

<table>
<thead>
<tr>
<th>Plating b</th>
<th>Media c</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
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</tr>
<tr>
<td>1</td>
<td>$3.2 \times 10^4$</td>
<td>$6.8 \times 10^4$</td>
<td>$9.6 \times 10^4$</td>
<td>$7.8 \times 10^4$</td>
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</tr>
<tr>
<td>2</td>
<td>$3.3 \times 10^4$</td>
<td>$6.0 \times 10^5$</td>
<td>$6.0 \times 10^5$</td>
<td>$1.9 \times 10^5$</td>
<td></td>
</tr>
</tbody>
</table>

aISP874 8325-4 rl-r2-m31+m32+ tyrB282::Tn551 lys-115 pig-131
ISP856 8325-4 (pC194).

bProtoplast-DNA mixture was either plated directly (method 1), or allowed to express for 3.5 hour at 30 C (method 2), before plating onto selective media.

cA = DM3 using chloramphenicol in an agar overlay; B = DM3 with direct incorporation of chloramphenicol; C = R-agar using chloramphenicol in an agar overlay; and D = R-agar with direct incorporation of chloramphenicol. Selection was made for resistance to 10 µg/ml of chloramphenicol. Frequencies are expressed as protoplast plasmid transformants recovered per µg of pC194 DNA.
plating the protoplast-DNA mixture immediately onto regeneration media (plating 1), with allowing a 3.5-hour expression period before plating (plating 2). Regardless of media and application of the selective agent, plating method 2 produced more pC194 transformants than plating method 1. From the data presented in Table 6, it was decided to plate putative plasmid-containing protoplasts following a 3.5-hour incubation at 30°C directly onto R-agar containing the selective agent. Furthermore, R-agar was considerably less complicated and less expensive to prepare than DM3 agar, which are additional advantages of using R-agar.

**Effect of selective levels of antibiotic, CsCl purification of plasmid DNA, and induction for gene expression on the frequency of protoplast plasmid transformation**

A background lawn of cells was observed on control and experimental plates when selection was made on R-agar containing 4 μg per ml of chloramphenicol, because *S. aureus* exhibits a basal-level resistance to this antibiotic. The background growth was eliminated by exposure to higher selective levels of chloramphenicol. To determine if higher selective levels of chloramphenicol would affect the recovery of transformants, plasmid pC194 was presented to protoplasts of ISP1047, and the resulting mixture was plated onto R-agar containing either 3 or 10 μg per ml of chloramphenicol. As shown in Table 7, the transformation frequency for pC194 was higher when selection was made for 10 μg per ml of chloramphenicol than when selection was made for 3 μg/ml. This higher frequency on R-agar plus 10 μg/ml of chloramphenicol was not affected either by antibiotic induction of the protoplast-DNA mixture or by CsCl
Table 7. Transformation of protoplasts of strain ISP1047 with plasmid DNA from strains ISP856 and ISP1578*

<table>
<thead>
<tr>
<th>Selection on:</th>
<th>Frequency(^b) of:</th>
<th>3 (\mu g/ml) Cm</th>
<th>10 (\mu g/ml) Cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>pC194(^d)</td>
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<td>4.76 (\times 10^4)</td>
<td>2.09 (\times 10^5)</td>
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<tr>
<td>pC194(^e)</td>
<td></td>
<td>5.99 (\times 10^4)</td>
<td>2.39 (\times 10^5)</td>
</tr>
<tr>
<td>pC194(^de)</td>
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<td>1.87 (\times 10^5)</td>
<td>3.08 (\times 10^5)</td>
</tr>
<tr>
<td>pTVl(_{ts})(^d)</td>
<td>1 (\mu g/ml) Em</td>
<td>7.72 (\times 10^4)</td>
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</tr>
<tr>
<td>pTVl(_{ts})(^de)</td>
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<td>9.13 (\times 10^4)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)ISP1047 80CR3 nov-142  
ISP856 8325-4 (pC194)  
ISP1578 8325-4 pig-131 (pTVl\(_{ts}\)).

\(^b\)Expressed as protoplast plasmid transformants recovered per \(\mu g\) of plasmid DNA.

\(^c\)Background lawn of cells present due to basal-level resistance to chloramphenicol. Significantly less background growth when selection was made for resistance to 10 \(\mu g/ml\) of chloramphenicol compared to 3 \(\mu g/ml\) of chloramphenicol.

\(^d\)Plasmid DNA was purified by CsCl density-gradient centrifugation.

\(^e\)Transformation mixtures were incubated for 3.5 h at 30 C in the presence of 0.1 \(\mu g/ml\) of chloramphenicol (pC194), or 0.1 \(\mu g/ml\) of erythromycin (pTVl\(_{ts}\)) prior to plating onto R-agar.
purification of pC194 DNA. Because use of 3 ug/ml of chloramphenicol yielded fewer transformants and resulted in a significant amount of background growth, 10 ug per ml of chloramphenicol was used in subsequent experiments to select for protoplast plasmid transformants.

Table 7 also presents data demonstrating that both CsCl-gradient purification of plasmid DNA and antibiotic induction for gene expression contributed to increased recovery of transformants. When selection was made on 3 ug/ml of chloramphenicol, almost 4 times as many transformants were obtained for the induced preparation, compared to the non-induced preparation of pC194 (1.87 x 10^5 vs 4.76 x 10^4). Non-CsCl purified pC194 DNA that was induced yielded slightly more transformants than a preparation of CsCl-purified pC194 that was not induced (5.99 x 10^4 vs 4.76 x 10^4). An induced preparation of CsCl-purified pC194 DNA produced more transformants (1.87 x 10^5) than non-CsCl purified and induced (5.99 x 10^4), or CsCl-purified not induced (4.76 x 10^4) preparations of pC194. This same trend was also observed when selection was made on R-agar containing 10 ug/ml of chloramphenicol: pC194 CsCl-purified and induced (3.08 x 10^5), yielded more transformants than pC194 non-CsCl-purified and induced (2.39 x 10^5), which in turn produced more transformants than pC194 CsCl-purified not induced (2.09 x 10^5).

Preparations of CsCl-purified plasmid pTV1ts were also presented to protoplasts of ISP1047 to determine if induction with 0.1 ug/ml of erythromycin would result in increased recovery of plasmid transformants. As shown in Table 7, the values obtained for an induced preparation of pTV1ts were higher than for a non-induced preparation of this same DNA
(9.13 \times 10^6 \text{ transformants per } \mu g \text{ of } \text{pTV1}_{ts} \text{ DNA when induced, compared to } 7.22 \times 10^4 \text{ per } \mu g \text{ when not induced}).

The data in Table 7 demonstrated that CsCl-purified plasmid DNA produced more transformants than non-CsCl purified plasmid DNA. Furthermore, more transformants were recovered from preparations induced for 3.5 h in the presence of the appropriate antibiotic, than from identical preparations of plasmid DNAs that were not induced. When selection was made for resistance to 10 \mu g/ml of chloramphenicol, the transformation frequency of an induced preparation of CsCl-purified pC194 DNA (frequency of 3.08 \times 10^5) was significantly higher than CsCl-purified and induced pTV1_{ts} DNA (frequency of 9.13 \times 10^4). Plasmid pC194 (2.9 kb) is considerably smaller than pTV1_{ts} (12.4 kb), and small DNAs typically transform more efficiently than large DNAs. The data in Table 7 support this generalization.

To determine if CsCl-purified DNA affected competent-cell transformation of E. coli, pJH101 was transformed into competent cells of E. coli strain HB101. Ampicillin-resistant transformants were obtained at a frequency of 1.10 \times 10^5 \text{ per } \mu g \text{ of CsCl-purified pJH101 DNA, compared to a frequency of } 1.35 \times 10^4 \text{ per } \mu g \text{ of non-CsCl-purified pJH101 DNA (data not shown). CsCl-purified plasmid DNA used in either protoplast plasmid transformation of S. aureus, or competent cell transformation of E. coli, resulted in higher frequencies of transformation than identical preparations of non-CsCl-purified plasmid DNAs.}
Effect of heat treatment of recipient protoplasts on homologous and heterologous protoplast plasmid transformation of *S. aureus*

Sjostrom et al. (1979) reported that preheated restrictive strains of competent *S. aureus* were transformed with heterologous plasmid DNA at frequencies comparable to those for modified homologous DNA. For the purposes of this study, it was important to determine if similar results could be obtained for *S. aureus* protoplast plasmid transformation. As shown in Table 8, protoplasts of a restrictive strain (ISP990) were transformed with both homologous plasmid DNA (pTVl<sub>CS</sub> and pI258 isolated from *S. aureus*) and heterologous plasmid DNA (pTV20 isolated from *B. subtilis*). Duplicate transformations were performed with each plasmid: one preparation of each plasmid was introduced to protoplasts of ISP990 that were incubated at 56 °C for 45 seconds prior to the addition of plasmid DNA. For transformation using heterologous DNA (pTV20), transformants were not obtained from the non-heated preparation, whereas transformants were obtained at a frequency of 3.0 x 10^2 when the protoplasts were heated. For transformation of ISP990 protoplasts with homologous DNA, the non-heated preparation of pTVl<sub>CS</sub> produced transformants at a frequency of 2.8 x 10^5, compared to 2.0 x 10^5 for the heated preparation. The slight difference in frequency between the non-heated and heated preparations of pTVl<sub>CS</sub> could be explained by loss of protoplasts as a consequence of the elevated temperature. For plasmid pI258, the heated preparation produced transformants at a frequency about 3.5 times greater than the non-heated preparation: 9.8 x 10^4 transformants per ug of pI258 DNA presented to heated protoplasts, compared to 2.8 x 10^4 transformants per ug of pI258 DNA.
Table 8. Transformation of protoplasts of strain ISP990 with plasmid DNA from strains ISP1769, ISP1578, and ISP479

<table>
<thead>
<tr>
<th>Em&lt;sup&gt;b&lt;/sup&gt; of:</th>
<th>Frequency&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>pTV20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>pTV20</td>
<td>0</td>
</tr>
<tr>
<td>pTV1&lt;sub&gt;ts&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>pTV1&lt;sub&gt;ts&lt;/sub&gt;</td>
<td>2.8 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>pI258&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.8 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>pI258</td>
<td>2.8 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>ISP990  8325  thrB<sub>106</sub>  tyrB<sub>282</sub>::Tn<sub>551</sub>  ermB<sub>321</sub>  trpE<sub>85</sub>  tmn-3106  uraA<sub>141</sub>  nov-142  mec-4916

ISP1769  B. subtilis  BD170 (pTV20)

ISP1578  8325-4  pig<sub>131</sub> (pTV1<sub>ts</sub>)

ISP479  8325-4  pig<sub>131</sub> (pI258).

<sup>b</sup>Expressed as protoplast plasmid transformants recovered per ug of CsCl-purified plasmid DNA. Transformation mixtures were incubated for 3.5 h at 30°C in the presence of 0.1 ug/ml of erythromycin prior to plating onto R-agar containing 1.5 ug/ml of erythromycin.

<sup>c</sup>Protoplasts were heated for 45 seconds at 56°C before plasmid DNA was added.
presented to protoplasts that were not heated (Table 8).

Plasmids pTV20 and pTV_{1Ts} contain the same Gram-positive origin of replication, the pEl94 origin, and are similar in size at 15.5 kb and 12.4 kb, respectively. Although pTV_{1Ts} and pTV20 are similar, pTV_{1Ts} transformed heated protoplasts at a frequency of $2.0 \times 10^5$ per ug of DNA, compared to a frequency of $3.0 \times 10^2$ per ug of pTV20. pTV_{1Ts} transformed non-heated protoplasts at a frequency of $2.8 \times 10^5$ per ug of DNA (see Table 8). When protoplasts were not heated, transformants were not obtained with pTV20 DNA. The data in Table 8 provided evidence that the origin of DNA ($B.\ subtilis$ vs $S.\ aureus$) largely determined the transformability of a particular plasmid. Restriction barriers were (partially) circumvented by heating $S.\ aureus$ protoplasts just prior to the addition of DNA.

The transfer frequency was significantly greater for pTV_{1Ts} compared to pI258, regardless of whether or not the protoplasts were heated (see Table 8). Plasmid pI258 (28.2 kb) is more than twice as large as pTV_{1Ts} (12.4 kb); therefore, it was not surprising that pTV_{1Ts} transformed ISP990 at a higher frequency than pI258. The transformation frequency for pI258 was increased when protoplasts were heated, suggesting that heat treatment of $S.\ aureus$ protoplasts may increase the permeability of the cell membrane, or inactivate a heat-sensitive nuclease, and allow for internalization of large plasmids.

**Protocol for maximum transformation frequencies of $S.\ aureus$ protoplasts**

The following conditions consistently yielded the highest frequencies of plasmid transformants via protoplast plasmid transformation:

CsCl-purified plasmid DNA was presented to protoplasts prepared via the
Stahl and Pattee protocol and maintained at 56°C for 45 seconds, and the resulting mixture was plated (following a 3.5-hour incubation at 30°C, in the presence of inducing levels of antibiotics if appropriate, to allow for gene expression) onto R-agar containing either 10 μg per ml of chloramphenicol or 1.5 μg per ml of erythromycin. Following an incubation at 30°C at high humidity for approximately 5 days, regenerated colonies were counted and replica-plated onto appropriate media to score for unselected plasmid markers. After incubation for approximately 30 h at 30°C, the replica plates were scored.

By utilizing the optimized protoplast transformation procedure detailed above, several additional attempts were made to transform pLUCH3 into protoplasts of different recipient strains of *S. aureus*. As with earlier efforts to introduce this vector into *S. aureus*, these experiments were not successful (data not shown). Although much useful information concerning the ideal conditions for introducing plasmid DNA into *S. aureus* was accumulated, it was not possible to introduce the integrable vector pLUCH3 into this organism. Based on information obtained from efforts to transform pLUCH3 into *S. aureus* protoplasts, a novel insertion vector, pPQ126, was constructed that was amenable for use in *S. aureus* (see below).

**Characterization of pTV1ts**

As described in the previous section, attempts to utilize an established integrable plasmid in *S. aureus*, a pJH101-based vector, were not successful. Because insertion plasmids do not replicate autonomously prior to integration, it was important to determine if pLUCH3 entered the cell and circumvented restriction barriers or if a problem existed at the
level of integration (reliance on selection for resistance to chloramphenicol to recover integrated plasmids). To gain insight into this problem, a temperature-sensitive insertion vector was introduced and established at the permissive temperature before selecting for integration into the host chromosome by shifting to the non-permissive temperature. Plasmid pTVl\textsubscript{ts} (12.4 kb) encodes for resistance to chloramphenicol (\textit{cat} gene from \textit{S. aureus} plasmid pC194) and erythromycin (Tn917). More importantly, this vector also harbors a mutated origin of replication, responsible for an abrupt block in plasmid replication at 38 C.

The effect of using chloramphenicol to select for transfer of pTVl\textsubscript{ts} into \textit{S. aureus} via protoplast plasmid transformation

In previous experiments, pTVl\textsubscript{ts} was utilized to define conditions for obtaining higher frequencies for protoplast plasmid transformation, and erythromycin was used to select for pTVl\textsubscript{ts}-containing transformants (see Tables 7 and 8). It was necessary, therefore, to determine the effect of selection for resistance to chloramphenicol on the recovery of pTVl\textsubscript{ts} transformants. The results comparing erythromycin to chloramphenicol for selection of pTVl\textsubscript{ts} transformants via protoplast plasmid transformation are shown in Table 9. Plasmids pC194 and pE194\textsubscript{ts} were utilized as control DNAs. Both pE194\textsubscript{ts} and pC194 transformed at good efficiency, but pE194\textsubscript{ts} transformed ISP990 about 1.5 times better than pC194 (3.96 x 10\textsuperscript{5} transformants per ug of pE194\textsubscript{ts} DNA compared to 2.35 x 10\textsuperscript{5} transformants per ug of pC194 DNA). Although somewhat similar in size, pC194 (2.9 kb) is about 800 base pairs smaller than pE194\textsubscript{ts} (3.7 kb). For this reason, one
Table 9. Transformation of protoplasts of strain ISP990 with plasmid DNA from strains ISP856, ISP1574, and ISP1578a

<table>
<thead>
<tr>
<th></th>
<th>Frequencyb of:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emr</td>
<td>Cmr</td>
</tr>
<tr>
<td>pE194ts</td>
<td>3.96 x 10^5</td>
<td>NDC</td>
</tr>
<tr>
<td>pC194</td>
<td>NDC</td>
<td>2.35 x 10^5</td>
</tr>
<tr>
<td>pTVlts</td>
<td>1.56 x 10^4</td>
<td>6.60 x 10^4</td>
</tr>
</tbody>
</table>

aISP990 8325 thrB106 tyrB282::Tn551 ermB321 trpE85 tmn-3106 uraA141 nov-142 mec-4916
ISP856 8325-4 (pC194)
ISP1574 8325-4 pig-131 (pE194ts)
ISP1578 8325-4 pig-131 (pTVlts).

bExpressed as protoplast plasmid transformants recovered per ug of CsCl-purified plasmid DNA. Transformation mixtures were incubated for 3.5 h at 30°C in the presence of 0.1 ug/ml of either erythromycin (pTVlts and pE194ts) or chloramphenicol (pTVlts and pC194) prior to plating onto R-agar containing either 1.5 ug/ml of erythromycin or 10 ug/ml of chloramphenicol. Protoplasts of ISP990 were not heated prior to the addition of plasmid DNA.

cND = not determined.
would expect pC194 might transform *S. aureus* protoplasts more efficiently than pE194ts. The observed difference in transformation frequency between pE194ts and pC194 may have resulted from loss of viable protoplasts because chloramphenicol may be more stressful than erythromycin on regenerating protoplasts.

When selection was made on R-agar containing 1.5 ug/ml of erythromycin, pTV1ts-containing transformants were recovered at a frequency of 1.56 x 10^4 per ug of DNA. In comparison, protoplasts of ISP990 were transformed with pE194ts DNA at a frequency about 25 times greater than the frequency reported in Table 9 for pTV1ts (3.96 x 10^5 pE194ts transformants vs 1.56 x 10^4 pTV1ts transformants). An explanation for the difference between the recovery of pE194ts and pTV1ts transformants on R-agar plus 1.5 ug/ml of erythromycin was the considerable size differential between these two plasmids (pE194ts is 3.7 kb and pTV1ts is 12.4 kb).

pTV1ts transformants were obtained at a frequency of 6.6 x 10^1 when the transformation mixture was plated on R-agar containing 10 ug/ml of chloramphenicol. The value 6.6 x 10^1 represents a significant reduction in pTV1ts transformants compared to the value of 1.56 x 10^4 obtained on R-agar plus 1.5 ug/ml of erythromycin. It was not clear, however, why chloramphenicol selection resulted in a decreased transformation frequency, because pC194 transformed protoplasts of ISP990 at good efficiency (2.35 x 10^5 transformants per ug of DNA), and because transformation of pTV1ts occurred at good efficiency on R-agar plus 1.5 ug/ml of erythromycin (1.56 x 10^4).
Phenotypic and molecular confirmation of pTV1\textsubscript{ts} deletions resulting from selection for chloramphenicol-resistant transformants via protoplast plasmid transformation

Representative transformants recovered for each of the plasmids transformed into ISP990 (see Table 9) were screened for resistance to chloramphenicol and erythromycin: isolates were replica-plated onto media containing chloramphenicol at concentrations of 4, 10, 15, 20, and 50 ug/ml, and erythromycin at concentrations of 10 and 25 ug/ml. The recipient for this experiment, ISP990, was resistant to about 4 ug/ml of chloramphenicol, but sensitive to 10 ug/ml each of chloramphenicol and erythromycin (data not shown). All 258 transformants of ISP990 containing pE194\textsubscript{ts} were resistant to 25 ug/ml of erythromycin, but sensitive to 10 ug/ml of chloramphenicol (data not shown). All 78 transformants that contained pC194 were resistant to 50 ug/ml of chloramphenicol, but sensitive to 10 ug/ml of erythromycin (data not shown).

All 87 pTV1\textsubscript{ts} transformants recovered from R-agar plus 1.5 ug/ml of erythromycin were resistant to about 15 ug per ml of chloramphenicol and 25 ug per ml of erythromycin (data not shown). Although basal-level resistance to chloramphenicol for ISP990 was about 4 ug/ml, transformants harboring pTV1\textsubscript{ts} were resistant to about 15 ug/ml of chloramphenicol. Thirty-one pTV1\textsubscript{ts}-containing transformants of ISP990 obtained using chloramphenicol selection were also screened for resistance to erythromycin and chloramphenicol. As shown in Table 10, the 31 transformants comprised three distinct phenotypic classes: class I isolates (64.5%) were resistant to 50 ug/ml of chloramphenicol, but sensitive to 10 ug/ml of erythromycin;
Table 10. Phenotypic distribution of pTV1<sub>TS</sub>-containing transformants from protoplast plasmid transformation of ISP990<sup>a</sup>

<table>
<thead>
<tr>
<th>Analysis of phenotypes</th>
<th>Antibiotic concentration (ug/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cm</td>
<td>15</td>
</tr>
<tr>
<td>Of 31 Cm&lt;sup&gt;R&lt;/sup&gt; transformants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64.5% (20)</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>19.4% (6)</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>16.1% (5)</td>
<td>r</td>
<td>+/-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Protoplasts of ISP990 were transformed with pTV1<sub>TS</sub> and plated onto R-agar plus 10 ug/ml of chloramphenicol. Transformants were recovered at a frequency of 6.6 x 10<sup>1</sup> per ug of pTV1<sub>TS</sub> DNA (Table 10).

<sup>b</sup>Transformants were replica-plated onto media containing different concentrations of either chloramphenicol or erythromycin. Following a 30 h incubation at 30 C, the plates were scored for growth (r = resistant), no growth (s = sensitive), or poor growth (+/-) of the transformants.
class II isolates (19.4%) were resistant to at least 15 μg/ml of chloramphenicol, but sensitive to 10 μg/ml of erythromycin; and class III isolates (16.1%) were resistant to about 15 μg per ml of chloramphenicol, and at least 10 μg/ml of erythromycin. From the data presented in Tables 9 and 10 for pTVl<sub>Ts</sub>, it was apparent that selection for chloramphenicol resistance resulted in reduction of the total number of transformants, and phenotypic variation was present among the transformants that were recovered. The most obvious explanation for the different phenotypes was that classes I and II from Table 10 contained forms of pTVl<sub>Ts</sub> that contained deletions. The erythromycin-sensitive phenotype of classes I and II was attributed to loss (deletion) of all or part of the em gene of pTVl<sub>Ts</sub>. Class III isolates were of the same phenotype of pTVl<sub>Ts</sub> transformants recovered by using selection for resistance to erythromycin, and were thought to contain an intact copy of pTVl<sub>Ts</sub>.

To determine if any or all classes described above contained deleted forms of pTVl<sub>Ts</sub>, plasmid DNA was extracted from representative isolates and analyzed by agarose-gel electrophoresis. All isolates from a particular class displayed the same migration pattern (data not shown); therefore, Figure 8 includes data for one member of each class. The results shown in Figure 8 confirmed that deleted forms of pTVl<sub>Ts</sub> were present in classes I and II, and verified the presence of intact copies of pTVl<sub>Ts</sub> in class III and the erythromycin-selected transformants (see below). Lane 2 contains a class III transformant (JBL14a; pTVl<sub>Ts</sub>), and lane 5 contains a transformant obtained via selection for resistance to erythromycin (JBL14b; pTVl<sub>Ts</sub>); the migration pattern of the plasmid DNA in lanes 2 and 5 are identical.
Figure 8. Analysis of \( pTV1_{ts} \) deletion derivatives obtained from protoplast plasmid transformation of ISP990 with selection for resistance to 10 \( \mu g/ml \) of chloramphenicol

Lane 1, \textit{HindIII}-digested lambda standard (0.30 \( \mu g \) total); lane 2, \( pTV1_{ts} \) isolated from JBL14a; lane 3, \( pLUCH11 \) isolated from JBL11; lane 4, \( pLUCH10 \) isolated from JBL10; lane 5, \( pTV1_{ts} \) isolated from JBL14b; lane 6, \textit{HindIII}-digested lambda standard (0.30 \( \mu g \) total).
JBL14a and JBL14b share the same phenotype and plasmid profile, and represent successful protoplast plasmid transformation of pTV1ts into ISP990. As shown in lane 4 of Figure 8, plasmid pLUCH10 (JBL10) was the product of a major deletion of pTV1ts; compare pLUCH10 in lane 4 to an intact copy of pTV1ts in lanes 2 and 5. Plasmid pLUCH11 (JBL11, lane 3) also suffered a deletion, but the deletion in pLUCH11 was not as extensive as the deletion in pLUCH10. Therefore, these data support the hypothesis that transformants JBL10 and JBL11 (class I and class II, respectively) are erythromycin sensitive as a consequence of deletion. Deletions may also explain why JBL10 and JBL11 were more resistant than transformants containing parental pTV1ts (e.g., JBL14a and JBL14b) to higher levels of chloramphenicol. Smaller plasmids typically exist in higher copy number than larger plasmids, and increased copy number also results in increased production of plasmid-encoded products (e.g., chloramphenicol acetyl transferase). The smallest deletion derivative (pLUCH10; 50 ug/ml) was resistant to higher levels of chloramphenicol than the next largest deletion derivative (pLUCH11; between 15 and 20 ug/ml), which was resistant to slightly higher levels of chloramphenicol than the parental plasmid (pTV1ts; 15 ug/ml).

Construction of pPQ126

To adapt pTV1ts for use as an insertion plasmid, it was necessary to inactivate Tn917 transposition functions and add a readily selectable marker. Digestion of pTV1 with HindIII produces fragments 11.1 and 1.3 kb in length; the smaller 1.3-kb fragment contains the information required for transposition of Tn917 (Perkins and Youngman, 1984). The strategy for
modifying pTV1cs was to remove the 1.3-kb fragment and replace it with another HindIII fragment containing a readily selectable marker. The gentamicin-resistance determinant of Tn4001 was chosen because it was known to reside on a 2.5-kb HindIII fragment, and it was readily selectable in S. aureus (Lyon et al., 1984; Tennent et al., 1985).

To facilitate the cloning of the gentamicin-resistant determinant, an insertion of Tn4001 was obtained in plasmid PQ58. Figure 9 shows the EcoRI and HindIII restriction analyses of plasmid PQ58. To avoid possible confusion, it should be mentioned that plasmid PQ58 is plasmid PI258 plus an insertion of Tn4001. The EcoRI-digestion patterns of PQ58 and PI258 in Figure 9 (lanes 1 and 3, respectively) are similar; however, the EcoA fragment of PQ58 is larger (migrates slower) than the EcoA fragment of PI258. Plasmids PI258 and PQ58 were digested with EcoRI, and the EcoA fragments for each plasmid were isolated from agarose. Lanes 2 and 4 of Figure 9 contain the isolated EcoA fragments of plasmids PI258 and PQ58, respectively. By comparing lanes 1 and 2 to lanes 3 and 4, it was concluded that Tn4001 resides on the largest (EcoA) fragment of EcoRI-digested PQ58. Lanes 7 and 8 contain the HindIII restriction analysis of the EcoA fragments only for plasmids PI258 (lane 7) and PQ58 (lane 8). For plasmid PQ58, two fragments were visible following digestion with HindIII; an 11.1-kb fragment (A, Figure 9), and a 1.3-kb fragment (E, Figure 9). Compared with the HindIII pattern of PI258 (lane 6), 2 additional fragments were observed (labeled C and D) and one original fragment, the HinB fragment, was absent from PQ58 (lane 9). The 2.8-md HinB fragment of PI258 contains the ermB gene of Tn551 and, therefore, the
Figure 9. Restriction characterization of plasmids pI258, pPQ58, and pTVl<sub>ts</sub>, and HindIII-digestion of the EcoA fragments only of plasmids pI258 and pPQ58

Lane 1, EcoRI-digested pI258; lane 2, EcoA fragment of pI258; lane 3, EcoRI-digested pPQ58; lane 4, EcoA fragment of pPQ58; lane 5, HindIII-digested lambda standard (0.30 ug total); lane 6, HindIII-digested pI258; lane 7, HindIII-digested pI258 EcoA fragment; lane 8, HindIII-digested pPQ58 EcoA fragment; lane 9, HindIII-digested pPQ58; lane 10, HindIII-digested pTVl<sub>ts</sub>; lane 11, HindIII-digested lambda standard (0.30 ug total).

A = 11.1 kb HindIII fragment of pTVl<sub>ts</sub>; B = doublet consisting of the HinA fragment of pPQ58 (5.1 kb; the HinB fragment of pI258 containing a portion of Tn4001) and the HinB fragment of pPQ58 (4.9 kb; the HinA fragment of pI258); C = 2.5 kb HindIII fragment of pPQ58 that encodes for gentamicin resistance; D = approximately 1.5 kb HinH fragment of pPQ58 (Tn4001 DNA); E = 1.3 kb HindIII fragment of pTVl<sub>ts</sub> (contains information required for transposition of Tn917).
HinB fragment is a component of the EcoA fragment of pI258. By comparing the migration patterns of the HindIII-digested EcoA fragments of pI258 (lane 7) and pPQ58 (lane 8), it was apparent that the largest Hin fragment of pPQ58 (labeled B in Figure 9) was actually a doublet, consisting of the HinA fragment of pPQ58 (approximately 5.1 kb; the HinB fragment of pI258 containing a portion of Tn4001), and the HinB fragment of pPQ58 (approximately 4.9 kb; the HinA fragment of pI258). From the HindIII and EcoRI analysis of plasmids pI258 and pPQ58 presented in Figure 9, it was concluded that Tn4001 had inserted onto the EcoA fragment of plasmid pI258 in close proximity to the HindIII site on the *erm*-distal end of the 2.8 md HinB fragment, producing plasmid pPQ58. The band in lanes 8 and 9 labelled C is the 2.5-kb HindIII fragment containing the gentamicin-resistance determinant of Tn4001. Figure 10 more clearly defines the location of Tn4001 on plasmid pPQ58 relative to other markers.

Cloning the 2.5 kb HindIII fragment of pPQ58 onto pTV1<sub>ES</sub>

Plasmid pPQ58 was digested with HindIII. The resulting fragments were ligated to HindIII-digested, CIP-treated pTV1<sub>ES</sub>. This ligation mixture was used to transform protoplasts of a restrictionless recipient strain of *S. aureus* (ISP1658) under conditions determined previously as optimum for protoplast plasmid transformation. Of 8 erythromycin-resistant transformants, only one was also resistant to 10 μg/ml of gentamicin and 10 μg/ml of chloramphenicol (data not presented). The Em<sup>e</sup>Gen<sup>c</sup>Cm<sup>c</sup> transformant was streaked for isolation and a single colony was retained for further study as ISP1844. Plasmid DNA was extracted from ISP1844, purified by CsCl centrifugation, and digested with HindIII for molecular identification (see...
Figure 10. Genetic and physical map of pPQ58 showing the approximate location of Tn4001.

Numbers are kilobase coordinates reading clockwise from the BamHI site at 8 o'clock. Heavy lines represent probable direction of transcription for the mer and asa operons.

Symbols represent restriction endonuclease sites: EcoRI, ●; XmaI, ▽; HpaI, ○; BglII, ○; XbaI, ●; BamHI, □; HindIII, □

(modified from Novick et al., 1979c).
Figure 11, lane 2). Lane 1 of Figure 11 contains HindIII-digested pTV1ts: both the 11.1-kb (labeled A), and the 1.3-kb (labeled C) fragments are clearly visible. Lanes 3 and 4 contain HindIII-digested pPQ58 and pI258, respectively. Lane 2 of Figure 11 demonstrates that ISP1844 contains a plasmid, designated pPQ126, composed of the 11.1-kb HindIII fragment of pTV1ts, plus the 2.5-kb HindIII fragment of plasmid pPQ58. Plasmid pPQ126 does not contain the 1.3-kb (labeled C) fragment of pTV1ts (the fragment essential for transposition of Tn917). A physical/genetic map of plasmid pPQ126 is given in Figure 12.

Characterization of pPQ126

Before pPQ126 was employed for use as an integrable plasmid in S. aureus, it was necessary to confirm that Tn917 and Tn4001 were transpositionally defective. Table 11 summarizes results of tests performed to investigate the ability of transposons Tn917, Tn551, and Tn4001 to transpose from various temperature-sensitive delivery vehicles. Youngman (1985) reported transposition frequencies of $5 \times 10^{-5}$ to $5 \times 10^{-4}$ for Tn917 on plasmid pTV1 in B. subtilis. Tn917 transposed at a frequency of $1.46 \times 10^{-3}$ from pTV1ts in S. aureus strain ISP1483 (see Table 11). The transposition frequency of $1.63 \times 10^{-4}$ for Tn551 from plasmid pI258 (Table 11), was in close agreement with already published results for transposition of Tn551 from this plasmid (Luchansky and Pattee, 1984; Novick et al., 1979b).

A translocation frequency of about $10^{-4}$ has also been reported for Tn4001 (Lyon et al., 1984). As shown in Table 11, Tn4001 transposed from plasmid pPQ61 in a recombination-deficient background (rec-; ISP1801) at
Figure 11. HindIII digestion of CsCl-purified preparations of plasmids pI258, pPQ58, pPQ126, and pTVlts

Lane 1, HindIII-digested pTVlts; lane 2, HindIII-digested pPQ126; lane 3, HindIII-digested pPQ58; lane 4, HindIII-digested pI258.

A = 11.1 kb Hin fragment of plasmids pTVlts and pPQ126; B = 2.5 kb Hin fragment of plasmids pPQ126 and pPQ58; C = 1.3 kb Hin fragment of pTVlts.

The 2.5 kb HindIII fragment contains the gentamicin resistance determinant from Tn4001, and the 1.3 kb HindIII fragment contains information essential for transposition of Tn917.
Figure 12. Genetic and physical map of pPQ126

Closed circles (○) position TaqI cleavage sites and open boxes (□) position SauIII(Mbo) cleavage sites. The approximate region required for replication (from plasmid pE194tg) is indicated by "pE194 rep". erm = the ribosome methyltransferase gene of Tn917, amp = the beta-lactamase gene of pBR322, cat = the chloramphenicol acetyl transferase gene of pC194, Gm = the 2.5 kb HindIII fragment of pPQ58 containing the gentamicin-resistance determinant of Tn4001. Arrows above antibiotic-resistance genes indicate direction of transcription (modified from Perkins and Youngman, 1984).
Table 11. Testing for transposition of Tn917, Tn551, and Tn4001

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Transposon</th>
<th>Antibiotic&lt;sup&gt;a&lt;/sup&gt; cfu&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Antibiotic&lt;sup&gt;a&lt;/sup&gt; cfu&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Transposition&lt;sup&gt;c&lt;/sup&gt; frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP1483</td>
<td>pTV1&lt;sub&gt;ts&lt;/sub&gt;</td>
<td>Tn917</td>
<td>2.02 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>3.05 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.46 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<tr>
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<td>pI258</td>
<td>Tn551</td>
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<td>1.63 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<td>pPQ61</td>
<td>Tn4001</td>
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<td>1.01 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8.95 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<tr>
<td>ISP1504</td>
<td>pPQ58</td>
<td>Tn551</td>
<td>2.02 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
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<td>3.59 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<tr>
<td>ISP1504</td>
<td>pPQ58</td>
<td>Tn4001</td>
<td>1.99 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>2.17 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISP1844</td>
<td>pPQ126</td>
<td>Tn917</td>
<td>8.50 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1.50 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>1.00 x 10&lt;sup&gt;4&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Cells from a fresh streak plate of each culture to be tested, previously grown at 30 C with selection, were harvested into 5 ml sterile saline, and serial dilutions were plated onto selective media as follows: BHI plus 10 ug/ml erythromycin for testing Tn917 and Tn551, and BHI plus 10 ug/ml gentamicin for testing Tn4001. Duplicate plates of each dilution were incubated at each temperature.

<sup>b</sup>Strains containing pPQ126 were incubated at 39 C, and strains containing plasmids pTV1<sub>ts</sub>, pI258, pPQ58, and pPQ61 were incubated at 43 C.

<sup>c</sup>Antibiotic-resistant cfu at 39 or 43 C divided by antibiotic-resistant cfu at 30 C. The values listed were corrected to include only the percent of cells at the high temperature that were phenotypically confirmed as plasmid-free while retaining the transposon.

<sup>d</sup>ND = not detected; less than the limits of detection.

<sup>e</sup>The reported value was an estimated cfu, based on the recovery of only 1 or 2 colonies per plate at a 10<sup>-4</sup> final dilution.
a frequency of $8.95 \times 10^{-4}$. Because the 2.5-kb gentamicin-resistance determinant of plasmid pPQ126 was originally harbored on plasmid DNA from ISP1504, the transpositional capability of Tn4001 from the parental plasmid, pPQ58, was examined. It was also possible to test for transposition of Tn551 in the same experiment. Tn551 transposed from plasmid pPQ58 at a frequency of $3.59 \times 10^{-4}$; however, it was not possible to detect transposition of Tn4001 from pPQ58. Efforts were not directed to determine why Tn4001 did not transpose from pPQ58 because only the gentamicin-resistance determinant from Tn4001 was essential to this study. Based on the behavior of Tn4001 on plasmid pPQ58, it was assumed that the 2.5-kb gentamicin-resistance Hind fragment of pPQ126 was incapable of transposition; the results in Table 11 support this contention. When selection was made on BHI plus 10 ug per ml of gentamicin at 39 C, transposition of the 2.5-kb HindIII fragment of pPQ126 was not detected.

Transposition of Tn917 from plasmid pPQ126 was not expected, because of the absence of the 1.3-kb HindIII fragment of Tn917 (Perkins and Youngman, 1984). As shown in Table 11, when cells of ISP1844 were plated on BHI plus 10 ug/ml of erythromycin and incubated at 39 C, it was not possible to detect transposition events of Tn917. For plasmid pPQ126 grown at 39 C on either erythromycin- or gentamicin-containing media and plated at a final dilution of $10^{-4}$, about 1 or 2 colonies were recovered along with a background haze of growth. These one or two colonies retained the phenotype of parental cells grown at 30 C (resistant to 10 ug/ml each of erythromycin and gentamicin, and 12 ug/ml chloramphenicol); however, the background haze was significantly diminished (data not shown). The 39 C
plates at the next highest dilution ($10^{-5}$) did not contain bona fide colonies and exhibited a reduction in the amount of background haze; the background haze was further diminished upon replica-plating to higher concentrations of antibiotics (data not shown). The background haze at low dilution on the erythromycin and gentamicin plates at 39 C was attributed to the total number of cells present; insufficient cell divisions to dilute out pPQ126 and/or to overcome phenotypic lag. A similar phenomenon was also observed for ISP1504 when selection was made for gentamicin-resistance at 43 C.

The results in Table 11 for ISP1844 also confirmed that pPQ126 retained the temperature-sensitive replication defect. Regardless of selection, a 5 to 6 log difference was observed between antibiotic-resistant cfu at 39 C and 30 C: erythromycin selection at 39 C ($1.5 \times 10^4$) revealed a difference of $10^5$ compared to 30 C ($8.5 \times 10^3$); gentamicin selection at 39 C ($1.0 \times 10^4$) showed a difference of $10^6$ compared to 30 C ($1.1 \times 10^{10}$). More importantly, pTV1$_{ts}$ (ISP1483) at 43 C yielded erythromycin-resistant cfu at $3.05 \times 10^6$, compared to $1.5 \times 10^4$ erythromycin-resistant cfu for pPQ126 (ISP1844) at 39 C. The reported difference between pTV1$_{ts}$ and pPQ126 was based on the recovery of only one or two colonies of ISP1844 per plate at a dilution of $10^{-4}$; therefore, the actual difference may be considerably greater.

Transfer of pPQ126 into recipient strains that contain homologous chromosomal DNA

The acquisition of pPQ126 from *S. aureus* (= ISP1844) enabled the use of transduction as the genetic transfer mechanism employed to move pPQ126
into recipient strains that contained homologous chromosomal DNA (e.g., Tn4001 or Tn551).

A phill lysate of ISP1844 (titer = 5.5 x 10^{10} pfu/ml) was prepared and used to transfer pPQ126 into various recipient strains. In the absence of chromosomal homology, pPQ126 was lost from a population of cells grown at 39 C (Table 11, ISP1844). To determine if pPQ126 would integrate into the host chromosome, provided that a chromosomal target was available, pPQ126 was transduced into ISP835 (= 8325-4 pig-131 trp-159::Tn551 ermB317) and ISP1565 (= 8325 pig-131 w[Chr::Tn4001]1210). pPQ126 was also transduced into a recipient containing a chromosomal copy of Tn4001 in a recombination-deficient background (= JBL122 8325-4 his-7 recA1 pig-131 w[Chr::Tn4001]2000) to determine if integration of pPQ126 was dependent on recA^+ function. Gentamicin-resistant pPQ126 transductants were recovered (per 10^9 cfu) at frequencies that ranged from 10 for pPQ126 transduced into ISP835 (= ISP1846), to 294 and 706 for pPQ126 transduced into ISP1565 (= JBL85) and JBL122 (= JBL177), respectively (data not presented). The results of experiments to test the behavior of pPQ126 in these genetic backgrounds are summarized below.

Testing for Integration of pPQ126 in ISP1844

In the absence of a chromosomal target (e.g., no chromosomal insertion of Tn551 or Tn4001), pPQ126 should be lost from a population of cells grown at elevated temperature. To test this hypothesis, pPQ126 was introduced into ISP1844, and a high-temperature, plasmid-elimination experiment was performed.
From the BHI plates without selection at 30 C, cfu were recovered at a frequency of $2.86 \times 10^9$ per ml (Table 12). Of the 36 isolates screened, 2 phenotypic classes were observed: about 6% (2) of the 36 isolates were resistant to only 6 ug/ml of chloramphenicol and 10 ug/ml each of erythromycin and gentamicin \([\text{Em}^\text{Gm}^\text{Cm}^\text{(6 ug/ml)}]\), and about 94% (34) of the isolates screened were resistant to 11 ug/ml of chloramphenicol and 10 ug/ml each of erythromycin and gentamicin \([\text{Em}^\text{Gm}^\text{Cm}^\text{(11 ug/ml)}]\). A representative member from each phenotypic class was retained for further analysis (JBL115 for resistance to only 6 ug/ml of chloramphenicol and JBL116 for resistance to 11 ug/ml of chloramphenicol). It was not clear why JBL115 was resistant to only 6 ug/ml of chloramphenicol. When BHI plates (without selection) were incubated at 39 C cfu were obtained at a frequency of $3.9 \times 10^9$ per ml. Of the 36 isolates screened, all displayed the \(\text{Em}^\text{Gm}^\text{Cm}^\text{(6 ug/ml)}\) phenotype. The integration frequency for plating on BHI (without selection) was reported as zero; no plasmid-containing cells, based on phenotype, were recovered at 39 C.

The \(\text{Em}^\text{Gm}^\text{Cm}^\text{(6 ug/ml)}\) selection plates produced cfu at a frequency of $3.18 \times 10^9$ at 30 C, and <1 x $10^4$ per ml at 39 C (see Table 12). The value <1 x $10^4$ was an estimated cfu, based on a haze of growth (no bona fide colonies) present on plates of a $10^{-4}$ dilution. All 32 isolates screened from the 30 C erythromycin-selection plates displayed the \(\text{pPQ126}\) phenotype \([\text{Em}^\text{Gm}^\text{Cm}^\text{(11 ug/ml)}]\). The haze from the 39 C plate \((10^{-4} \text{ dilution})\) was plasmid-free \([\text{Em}^\text{Gm}^\text{Cm}^\text{(6 ug/ml)}]\). A single isolate was retained from the erythromycin-selection plate at 30 C as JBL117. Although it was not possible to recover an isolated colony from the 39 C plate, a loopful of
Table 12. Testing for integration of pPQ126 in ISP1844<sup>a,b</sup>

<table>
<thead>
<tr>
<th>Selection for</th>
<th>PhenoType&lt;sup&gt;c&lt;/sup&gt; (no. scored)</th>
<th>Strain&lt;sup&gt;d&lt;/sup&gt;</th>
<th>PhenoType&lt;sup&gt;c&lt;/sup&gt; (no. scored)</th>
<th>Strain&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Integration frequency&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.8 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>36 A (2)</td>
<td>3.9 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>36 B (all)</td>
<td>JBL119 0</td>
</tr>
<tr>
<td>Em&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>32 C (all)</td>
<td>&lt;1 x 10&lt;sup&gt;4&lt;/sup&gt;,f</td>
<td>haze B</td>
<td>JBL117(a) 0</td>
</tr>
<tr>
<td>Gm&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.6 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>19 C (all)</td>
<td>4.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>80 D (all)</td>
<td>JBL120 JBL121 0</td>
</tr>
</tbody>
</table>

<sup>a</sup>ISP1844 = 8325-4 r<sup>-</sup> (pPQ126).

<sup>b</sup>Cells from a fresh streak plate of ISP1844, previously grown with selection at 30 C, were harvested into 5 ml sterile saline; serial dilutions were plated onto BHI + 10 µg/ml gentamicin or BHI + 10 µg/ml of erythromycin, or BHI. Duplicate plates at each dilution were incubated at each temperature.

<sup>c</sup>A = Em<sup>f</sup>Gm<sup>e</sup>Cm<sup>f</sup>(6ug/ml), B = Em<sup>g</sup>Gm<sup>e</sup>Cm<sup>f</sup>(6ug/ml), C = Em<sup>f</sup>Gm<sup>e</sup>Cm<sup>f</sup>(1ug/ml), D = Em<sup>g</sup>Gm<sup>e</sup>Cm<sup>f</sup>(6ug/ml).

<sup>d</sup>Designation for representative member of each phenotype retained for further analysis.

<sup>e</sup>cfu at 39 C divided by cfu at 30 C for each selection. The frequency was corrected to include only the percentage of cells recovered at 39 C that were confirmed phenotypically as plasmid free.

<sup>f</sup>An estimated cfu; background haze of growth on plate of 10<sup>-4</sup> dilution (no colonies).
growth was retained for further analysis as JBL117(a). The integration frequency for erythromycin selection was zero: pPQ126-containing cells (based on phenotype) were not detected at 39 C.

A value of $2.6 \times 10^9$ cfu per ml was observed for ISP1844 incubated at 30 C on BHI medium containing 10 ug per ml of gentamicin. All 19 isolates screened from this plating displayed the phenotype for autonomous pPQ126 ([Em^2Gm^2Cm^2(11 ug/ml)]; one isolate, designated JBL118, was retained for further study. The gentamicin plates incubated at 39 C yielded cfu at a frequency of $4.0 \times 10^5$ (2 plates of dilution $10^{-5}$, with 53 and 27 colonies each). All 80 isolates recovered at 39 C were Em^2Gm^2Cm^2(6 ug/ml), suggesting the absence of pPQ126 from these cultures. Two isolates, designated JBL120 and JBL121, were retained for further analysis. It was not clear why colonies were recovered on gentamicin-selection plates. In a previous experiment, 1 or 2 colonies per plate were recovered at a final dilution of $10^{-4}$ when cells of ISP1844 were plated on gentamicin and incubated at 39 C (see Table 11). Because all 80 isolates were Em^2 and resistant to only 6 ug/ml of chloramphenicol, the integration frequency was reported as zero.

To screen for plasmid pPQ126, DNA was isolated from JBL115 through JBL121, digested with HindIII, and analyzed by agarose gel electrophoresis. As discussed below, the agarose gel data (data not presented) coincided with the phenotypic data presented in Table 12. The Em^2Gm^2Cm^2(11 ug/ml) isolates from Table 12 contained pPQ126, based on their HindIII pattern (JBL116, JBL117, and JBL118). The Em^2Gm^2Cm^2(6 ug/ml) isolate (JBL115) produced 2 faint bands, corresponding to the HindIII fragments of pPQ126.
The data suggest that pPQ126 was partially eliminated from JBL115 grown at 30°C in the absence of selection; fewer copies of pPQ126 could also explain the lower level of resistance to chloramphenicol. The isolates phenotypically confirmed as missing pPQ126 [Em^Gm^Cm^(6 ug/ml)] or Em^Gm^Cm^(6 ug/ml)], were also shown by agarose gel analysis to lack this plasmid (JBL117(a), JBL119, JBL120, and JBL121). The phenotypic and molecular data demonstrated that in the absence of a chromosomal target (ISP1844), pPQ126 was lost from a population of cells under non-permissive conditions (39°C). Isolates missing pPQ126 were sensitive to 10 ug/ml each of erythromycin and gentamicin, and resistant to 6 ug/ml of chloramphenicol.

Testing for integration of pPQ126 in JBL85

Plasmid pPQ126 was transduced into ISP1565 to generate JBL85: pPQ126 in a recipient strain with a chromosomal target (w[Chr::Tn4001]1210). A high-temperature, plasmid-elimination experiment was performed with JBL85 to test for integration of pPQ126 into the chromosomal Tn4001 target. Without selection, cfu were recovered at 7.25 x 10^8 per ml at 30°C, and 2.45 x 10^8 per ml at 39°C (Table 13). All 60 isolates from the 30°C plates displayed the phenotype of autonomous pPQ126 [Em^Gm^Cm^(11 ug/ml)], and all 36 isolates from the 39°C plates appeared to lack pPQ126 [Em^Gm^Cm^(6 ug/ml)]. Two strains, JBL101 and JBL108, were isolated from the 30°C and 39°C plates, respectively, and retained for further analysis. Agarose gel electrophoresis of DNA extracted from JBL101 demonstrated the presence of pPQ126, whereas DNA extracted from JBL108 did not reveal the presence of pPQ126 (see below). Based on the phenotypic and molecular data, the
Table 13. Testing for integration of pPQ126 in JBL85\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Selection for</th>
<th>Phenotype\textsuperscript{c}</th>
<th>Strain\textsuperscript{d}</th>
<th>cfu at 39 C (no. scored)</th>
<th>Phenotype\textsuperscript{c}</th>
<th>Strain\textsuperscript{d}</th>
<th>Integration frequency\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.2 x 10\textsuperscript{8}</td>
<td>60 A (all)</td>
<td>JBL101</td>
<td>2.5 x 10\textsuperscript{8}</td>
<td>36 B (all)</td>
<td>JBL108</td>
</tr>
<tr>
<td>Em\textsuperscript{f}</td>
<td>7.6 x 10\textsuperscript{8}</td>
<td>85 A (all)</td>
<td>JBL107</td>
<td>4.4 x 10\textsuperscript{7}</td>
<td>53 A (all)</td>
<td>JBL113</td>
</tr>
</tbody>
</table>

\textsuperscript{a}JBL85 = 8325 pig-131 w[Chr::Tn4001]1210 (pPQ126).

\textsuperscript{b}Cells from a fresh streak plate of JBL85, previously grown with selection at 30 C, were harvested into 5 ml sterile saline; serial dilutions were plated onto BHI + 10 \mu g/ml of erythromycin, or BHI. Duplicate plates at each dilution were incubated at each temperature.

\textsuperscript{c}A = Em\textsuperscript{f}Gm\textsuperscript{f}Cm\textsuperscript{f}(11\mu g/ml), B = Em\textsuperscript{g}Gm\textsuperscript{f}Cm\textsuperscript{f}(6\mu g/ml).

\textsuperscript{d}Designation for representative member of each phenotype retained for further analysis.

\textsuperscript{e}cfu at 39 C divided by cfu at 30 C for each selection. The frequency was corrected to include only the percentage of cells recovered at 39 C that were confirmed phenotypically as plasmid free.
integration frequency for pPQ126 in JBL85 (without selection) was nil.

Em$^r$ cfu were obtained at a frequency of $7.55 \times 10^8$ per ml at 30 C, and at a frequency of $4.35 \times 10^7$ per ml when plates were incubated at 39 C on BHI plus 10 ug/ml of erythromycin. Of 85 isolates grown at 30 C and examined for the presence of JBL85, all displayed the appropriate phenotype for cells containing autonomous pPQ126 [Em$^r$Gm$^r$Cm$^r$(11 ug/ml)]; an isolate, JBL107, was retained for further study. A total of 53 Em$^r$ isolates from plates incubated at 39 C contained pPQ126, based on their Em$^r$Gm$^r$Cm$^r$(11 ug/ml) phenotype. Two isolates, JBL113 and JBL114, were retained for electrophoretic analysis. DNA was isolated from JBL101, JBL107, JBL108, JBL113, and JBL114, and digested with HindIII. The 30 C isolates (JBL101 and JBL107) contained autonomous pPQ126, and the 39 C isolates (JBL108 and JBL113 and JBL114) did not contain autonomous pPQ126 (see below). Because both Em$^r$ isolates from 39 C incubation (JBL113 and JBL114) displayed the pPQ126 phenotype but did not contain autonomous plasmid, the integration frequency for pPQ126 in JBL85 was reported as $5.76 \times 10^{-2}$ for erythromycin selection. In the absence of selection at 39 C, pPQ126 was lost from JBL108, and the level of resistance to chloramphenicol dropped to 6 ug/ml.

Testing for integration of pPQ126 in JBL177

After confirming that integration of pPQ126 occurred only in the presence of a chromosomal target, I decided to determine if integration was also dependent on host recombination function (rec$^+$). pPQ126 was transduced into a rec$^-$ recipient that contained a chromosomal copy of Tn4001 (= JBL177), and a high-temperature, plasmid-elimination experiment was conducted. Table 14 lists results obtained to determine the effect of
Table 14. Testing for integration of pPQ126 in JBL177

<table>
<thead>
<tr>
<th>Selection for</th>
<th>Phenotype at 30°C (no. scored)</th>
<th>Strain</th>
<th>Phenotype at 39°C (no. scored)</th>
<th>Strain</th>
<th>Integration frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.3 x 10^9 A (5), B (66)</td>
<td>JBL200</td>
<td>4.7 x 10^9 A (all)</td>
<td>JBL205</td>
<td>0</td>
</tr>
<tr>
<td>Em^R</td>
<td>6.9 x 10^9 B (all)</td>
<td>JBL204</td>
<td>8.4 x 10^4 C (all)</td>
<td>JBL207</td>
<td>1.2 x 10^-5</td>
</tr>
<tr>
<td>Gm^R</td>
<td>6.1 x 10^9 A (7), B (61)</td>
<td>JBL202</td>
<td>4.4 x 10^9 A (all)</td>
<td>JBL206</td>
<td>0</td>
</tr>
</tbody>
</table>

^aJBL177 = 8325-4 his-7 recA1 pig-131 w[Chr::Tn4001]2000 (pPQ126).

^bCells from a fresh streak plate of JBL177, previously grown with selection at 30°C, were harvested into 5 ml sterile saline; serial dilutions were plated onto BHI + 10 ug/ml gentamicin, or BHI + 10 ug/ml of erythromycin, or BHI. Duplicate plates at each dilution were incubated at each temperature.

^cA = Em^R Gm^R Cm^R (6ug/ml), B = Em^R Gm^R Cm^R (14ug/ml), C = Em^R Gm^R Cm^R (10ug/ml).

^dDesignation for representative member of each phenotype retained for further analysis.

^ecfu at 39°C divided by cfu at 30°C for each selection. The frequency was corrected to include only the percentage of cells recovered at 39°C that were confirmed phenotypically as plasmid free.
the rec^- phenotype on the integration of pPQ126.

Plates incubated at 30 C without selection yielded $7.3 \times 10^9$ cfu per ml; about 7% (5 of 71) were Em^Gm^Cm^(6 ug/ml), and about 93% (66 of 71) were Em^Gm^Cm^(14 ug/ml). One isolate, designated JBL200, was retained from the Em^ class [Em^Gm^Cm^(6 ug/ml)], and one isolate (JBL201) was retained from the Em^ class [Em^Gm^Cm^(14 ug/ml)]. From plates incubated at 39 C in the absence of selection, cfu were recovered at a frequency of $4.71 \times 10^9$ per ml. All 471 isolates screened were of the same phenotype [Em^Gm^Cm^(6 ug/ml)]; JBL205 was an isolate retained for further analysis. Based on phenotype, it was assumed that JBL200 and JBL205 had lost pPQ126, and that JBL201 retained this plasmid.

The data for gentamicin-selection with JBL177 were similar to the data obtained without selection. At 30 C, $6.1 \times 10^9$ cfu per ml were recovered. Of 68 gentamicin-resistant isolates tested, approximately 10% (7 of 68 total) were Em^ and resistant to only 6 ug/ml of chloramphenicol, and about 90% (61 of 68 total) were Em^ and resistant to 14 ug/ml of chloramphenicol. JBL202 and JBL203 were retained to represent the Em^ and Em^ classes, respectively. Gentamicin resistant cfu were recovered at a frequency of $4.4 \times 10^9$ per ml at 39 C. Of 440 isolates screened from 39 C masters, all were Em^ and resistant to 6 ug/ml of chloramphenicol; JBL206 was the single isolate retained from this class. By using only phenotypic data, JBL202 and JBL206 were assumed to lack pPQ126 and JBL203 was assumed to contain pPQ126.

When erythromycin-selection was used, about a 5 log difference was obtained between the cfu recovered at 30 C ($6.95 \times 10^9$) compared to 39 C
A single phenotype [Em^Gm^Cm^(14 ug/ml)] was displayed by all 70 isolates screened from 30 C masters (JBL204, Table 14, was representative of this class). Of 90 isolates screened from the 39 C masters, all displayed the phenotype Em^Gm^Cm^(10 ug/ml); two isolates, JBL207 and JBL208, were retained for further analysis. Compared to the Em^S and low-level chloramphenicol resistance exhibited by both JBL205 and JBL206, JBL207 and JBL208 were Em^R and resistant to 10 ug/ml of chloramphenicol. The phenotype of JBL207 and JBL208 suggested that pPQ126 had been retained in these two isolates.

DNA was extracted from isolates JBL200 through JBL208 and digested with HindIII. The data in Figure 13 are in agreement with the predictions made from the phenotypic data for determining whether a given strain harbored pPQ126. Lanes 3 and 5 contained the 30 C Em^S isolates JBL200 and JBL202; these lanes do not contain pPQ126. Lanes 4, 6, and 7 in Figure 13 contained the 30 C Em^R isolates JBL201, JBL203, and JBL204, respectively; regardless of selection, these isolates contained pPQ126 (compare to lane 2). Autonomous plasmid DNA was not evident in isolates JBL205, JBL206, JBL207, or JBL208 (lanes 8 to 11). JBL207 and JBL208, however, are Em^Cm^(10 ug/ml), suggesting that pPQ126 is present as an integrated plasmid in these isolates. By using the phenotypic and molecular data, the integration frequency for pPQ126 in a rec^7 background was determined as 1.2 x 10^-5 (erythromycin selection). It was not possible to detect integration of pPQ126 at an observable frequency on BHI without selection, or BHI plus 10 ug per ml of gentamicin. Although detectable, the integration of pPQ126 in JBL77 (1.2 x 10^-5) occurred at a frequency at least 3 orders of
Figure 13. Analysis of isolates obtained following a high-temperature plasmid-elimination experiment with JBL177

Lane 1, HindIII-digested lambda standard (0.30 ug total); lane 2, HindIII-digested JBL85 DNA (30 C, gec); lane 3, HindIII-digested JBL200 DNA (30 C, no selection); lane 4, HindIII-digested JBL201 DNA (30 C, no selection); lane 5, HindIII-digested JBL202 DNA (30 C, g); lane 6, HindIII-digested JBL203 DNA (30 C, g); lane 7, HindIII-digested JBL204 DNA (30 C, e); lane 8, HindIII-digested JBL205 DNA (39 C, no selection); lane 9, HindIII-digested JBL206 DNA (39 C, g); lane 10, HindIII-digested JBL207 DNA (39 C, e); lane 11, HindIII-digested JBL208 DNA (39 C, e); lane 12, HindIII-digested lambda standard (0.30 ug total).

All DNAs were extracted via the rapid preparation method from cells grown overnight in 10 ml of BHI broth and incubated at the specified temperature without selection, or in the presence of 3 ug/ml of gentamicin (g) or 3 ug/ml of erythromycin (e), or 3 ug/ml each of gentamicin and erythromycin and 1 ug/ml of chloramphenicol (gec).
magnitude lower than the integration of pPQ126 in JBL85 (5.76 x 10^-2); Table 13. The results obtained for JBL177 suggest that integration of pPQ126 is highly dependent upon host recombination functions.

Testing for integration of pPQ126 in ISP1846

To determine the frequency of integration of pPQ126 into a chromosomal Tn551 target, pPQ126 was transduced into ISP835 [ ISP1846 8325-4 pig-131 trp-159::Tn551 ermB317 (pPQ126)]. The results of a high-temperature, plasmid-elimination experiment using ISP1846 are presented in Table 15. Without selection, cfu were recovered at a frequency of 1.4 x 10^10 and 1.51 x 10^10 at 30 C and 39 C, respectively. From 30 C, approximately 8% (11) of the 142 isolates tested were Em^R Gm^S and resistant to only 6 ug/ml of chloramphenicol (JBL301), and about 92% (131 of 142) were Em^R Gm^R and resistant to 12 ug/ml of chloramphenicol (JBL300). Of the 153 isolates screened from the 39 C plates, all displayed the Em^R Gm^R Cm^R (6 ug/ml) phenotype: one isolate, JBL302, was retained for further analysis. The phenotype of JBL301 and JBL302 suggested that pPQ126 was missing from these isolates.

When selection was made for gentamicin resistance, isolates were recovered from 30 C plates at 1.29 x 10^10 cfu per ml, and from 39 C plates at 4.2 x 10^5 cfu per ml. The 30 C isolates were all Em^F Gm^R Cm^R (12 ug/ml); 2 isolates, JBL310 and JBL311, were retained. All 84 isolates tested from 39 C selection expressed the phenotype Em^F Gm^R Cm^F (10 ug/ml): 2 isolates were retained for further study (JBL314 and JBL315).

When selection was made for erythromycin resistance, cfu were recovered at a frequency of 1.42 x 10^10 from plates incubated at 30 C, and...
Table 15. Testing for integration of pPQ126 in ISP1846\(^a\),\(^b\)

<table>
<thead>
<tr>
<th>Selection for</th>
<th>Phenotype(^c)</th>
<th>Strain(^d)</th>
<th>Phenotype(^c)</th>
<th>Strain(^c)</th>
<th>Integration frequency(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cfu at 30 C (no. scored)</td>
<td></td>
<td>cfu at 39 C (no. scored)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>$1.4 \times 10^{10}$</td>
<td>142 A (131)</td>
<td>JBL300</td>
<td>153 B (all)</td>
<td>JBL302</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B (11)</td>
<td>JBL301</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Em(^f)</td>
<td>$1.4 \times 10^{10}$</td>
<td>113 A (all)</td>
<td>JBL304</td>
<td>297 C (all)</td>
<td>JBL306</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>JBL305</td>
<td></td>
<td>JBL307</td>
</tr>
<tr>
<td>Gm(^f)</td>
<td>$1.3 \times 10^{10}$</td>
<td>124 A (all)</td>
<td>JBL310</td>
<td>84 C (all)</td>
<td>JBL314</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>JBL311</td>
<td></td>
<td>JBL315</td>
</tr>
</tbody>
</table>

\(^a\)ISP1846 = 8325-4 pig-131 trp-159[Chr::Tn551 ermB317] (pPQ126).

\(^b\)Cells from a fresh streak plate of ISP1846, previously grown with selection at 30 C, were harvested into 5 ml sterile saline; serial dilutions were plated onto BHI + 10 µg/ml gentamicin, or BHI + 10 µg/ml of erythromycin, or BHI. Duplicate plates at each dilution were incubated at each temperature.

\(^c\)A = Em\(^f\)Gm\(^f\)Cm\(^f\)(12ug/ml), \(B = Em\boldsymbol{g}\Gm\boldsymbol{g}\Cm\boldsymbol{g}(6ug/ml), \(C = Em\boldsymbol{f}\Gm\boldsymbol{f}\Cm\boldsymbol{f}(10ug/ml).\)

\(^d\)Designation for representative member of each phenotype retained for further analysis.

\(^e\)cfu at 39 C divided by cfu at 30 C for each selection. The frequency was corrected to include only the percentage of cells recovered at 39 C that were confirmed phenotypically as plasmid free.
at a frequency of $3.13 \times 10^8$ cfu from plates incubated at 39 C. Two isolates each were retained from plates incubated at 30 C (JBL304 and JBL305) and 39 C (JBL306 and JBL307). All 113 isolates screened from 30 C plates were Em$^G$m$^C$m$^F$(12 ug/ml), and all 297 isolates screened from 39 C plates were Em$^G$m$^C$m$^F$(10 ug/ml).

The 39 C phenotype for either gentamicin-resistant or erythromycin-resistant isolates [Em$^G$m$^C$m$^F$(10 ug/ml)] was presumptive evidence for integration of pPQ126. BHI plates without selection at 39 C were Em$^G$m$^C$m and resistant to only 6 ug/ml of chloramphenicol. To further test for the presence or absence of pPQ126, DNA was extracted from the following isolates: JBL300, JBL301, JBL302, JBL303, JBL304, JBL305, JBL306, JBL307, JBL310, JBL311, JBL314, and JBL315. Following digestion of these preparations of DNA with HindIII, samples were analyzed by gel electrophoresis for the presence of pPQ126 fragments. Isolates presumed to contain integrated pPQ126, based on phenotype (JBL306, JBL307, JBL314, and JBL315), were also shown to be missing autonomous pPQ126 by electrophoretic analysis (data not shown). Although JBL302 was also missing pPQ126 (data not presented), the phenotype of this isolate [Em$^G$m$^C$m$^F$(6 ug/ml)] suggested loss of pPQ126 rather than integration. The 30 C isolates that displayed the autonomous pPQ126 phenotype yielded pPQ126 HindIII fragments: JBL300, JBL304, JBL305, JBL310, and JBL311 (data not presented).

Using both phenotypic data (Table 15) and molecular data, the integration frequency of pPQ126 in a Tn551 target was calculated to be $2.2 \times 10^{-2}$ for erythromycin selection, and $4.2 \times 10^{-5}$ for gentamicin selection.
Testing for transposition of Tn917 in ISP1578

For comparative purposes, a high-temperature, plasmid-elimination experiment was performed using ISP1578 to obtain phenotypic and gel electrophoretic evidence for transposition of Tn917, and elimination of pTVl<sub>ts</sub>. The results of this experiment are presented in Table 16. Without selection, 1.06 x 10<sup>10</sup> cfu per ml were recovered at 30°C, and 9.85 x 10<sup>9</sup> cfu per ml were recovered at 39°C. All 102 isolates screened from the 30°C plates displayed the phenotype for autonomous pTVl<sub>ts</sub> [Em<sup>R</sup>Cm<sup>R</sup>(12 ug/ml)]. All 112 isolates screened from the 39°C plates displayed the pTVl<sub>ts</sub>-minus phenotype [Em<sup>S</sup>Cm<sup>S</sup>(6 ug/ml)]. JBL316 and JBL317 were retained to represent isolates obtained without selection at 30°C and 39°C, respectively.

When selection was made for resistance to 10 ug/ml of erythromycin (see Table 16), 8.35 x 10<sup>9</sup> cfu per ml were recovered at 30°C and 2.03 x 10<sup>7</sup> cfu per ml were recovered at 39°C. Of 84 Em<sup>R</sup> isolates tested from 30°C masters, all were resistant to 10 ug/ml of erythromycin and 12 ug/ml of chloramphenicol; one isolate was retained (JBL319). Of 181 isolates screened from the 39°C erythromycin-selection plates, all were Em<sup>R</sup>Cm<sup>R</sup>(6 ug/ml); a single isolate, designated JBL320, was retained.

DNA was isolated from JBL316, JBL317, JBL319, and JBL320. Following digestion with HindIII, the DNA was analyzed electrophoretically. The 30°C isolates (JBL316 and JBL319) contained pPQ126, whereas this plasmid was not present in the 39°C isolates (JBL317 and JBL320) [data not presented]. By using gel electrophoresis data and phenotypic data (Table 16), the transposition frequency of Tn917 in ISP1578 was determined as 2.43 x 10<sup>-3</sup>. Although this value is slightly higher than the published transposition
Table 16. Testing for transposition of Tn917 in ISP1578<sup>a,b</sup>

<table>
<thead>
<tr>
<th>Selection for</th>
<th>Phenotype&lt;sup&gt;c&lt;/sup&gt; Strain&lt;sup&gt;d&lt;/sup&gt;</th>
<th>cfu at 30 C (no. scored)</th>
<th>Phenotype&lt;sup&gt;c&lt;/sup&gt; Strain&lt;sup&gt;d&lt;/sup&gt;</th>
<th>cfu at 39 C (no. scored)</th>
<th>Integration frequency&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.06 x 10&lt;sup&gt;10&lt;/sup&gt; JBL316</td>
<td>102 A (all)</td>
<td>9.85 x 10&lt;sup&gt;9&lt;/sup&gt; JBL317</td>
<td>112 B (all)</td>
<td>0</td>
</tr>
<tr>
<td>Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td>8.35 x 10&lt;sup&gt;9&lt;/sup&gt; JBL319</td>
<td>84 A (all)</td>
<td>2.03 x 10&lt;sup&gt;7&lt;/sup&gt; JBL320</td>
<td>181 C (all)</td>
<td>2.43 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>ISP1578 = 8325-4 pig-131 (pTV1<sub/ts</sub>.

<sup>b</sup>Cells from a fresh streak plate of ISP1578, previously grown with selection at 30°C, were harvested into 5 ml sterile saline; serial dilutions were plated onto BHI + 10 μg/ml of erythromycin, or BHI. Duplicate plates at each dilution were incubated at each temperature.

<sup>c</sup>A = Em<sup>r</sup>Cm<sup>r</sup>(12ug/ml), B = Em<sup>r</sup>Cm<sup>r</sup>(6ug/ml), C = Em<sup>r</sup>Cm<sup>r</sup>(6ug/ml).

<sup>d</sup>Designation for representative member of each phenotype retained for further analysis.

<sup>e</sup>cfu at 39°C divided by cfu at 30°C for each selection. The frequency was corrected to include only the percentage of cells recovered at 39°C that were confirmed phenotypically as plasmid free.
frequency for Tn917 ($5 \times 10^{-5}$ to $5 \times 10^{-4}$; Youngman, 1985), it compares closely to the frequency of $1.46 \times 10^{-3}$ for transposition of Tn917 in ISP1483 (Table 11).

Table 17 summarizes results from the experiments testing for integration of pPQ126 in the presence of homologous chromosomal target sequences (e.g., ISP1846 and JBL85) and/or in a rec<sup>+</sup> background (JBL177). Table 17 also contains results from experiments testing a situation where pPQ126 should not integrate (ISP1844), and testing the transposition frequency of Tn917 from pTVl<sub>TS</sub> (ISP1578).

The use of high molecular weight DNA isolated from ISP1846 for the isolation and transfer of chromosomally integrated pPQ126

Previous data demonstrated that growth of ISP1846 at 39 C, in the presence of either erythromycin or gentamicin, resulted in chromosomal integration of plasmid pPQ126 (see Table 15). To confirm that pPQ126 was integrated, high molecular weight DNA was prepared from cells of ISP1846 grown at 43 C, and was used to transform competent cells of ISP5. As shown previously (Table 4), competent cells of S. aureus did not readily internalize plasmid molecules; therefore, Em<sup>R</sup>Gm<sup>R</sup>Cm<sup>R</sup> transformants would only result from transfer of chromosomally integrated, not autonomous, pPQ126. As shown in Table 18, several Em<sup>R</sup>Gm<sup>R</sup>Cm<sup>R</sup> transformants were recovered following transformation of ISP5 with DNA prepared from ISP1846: all incubations for this transformation were performed at 39 C. Of 33 Em<sup>R</sup> transformants recovered, class A (12 of 33) and class H (1 of 33) demonstrated transfer of pPQ126 markers only [Em<sup>R</sup>Gm<sup>R</sup>Cm<sup>R</sup>(9 ug/ml)]. All other classes showed transfer of pPQ126 markers [Em<sup>R</sup>Gm<sup>R</sup>Cm<sup>R</sup>(9 ug/ml)], and
Table 17. Summary of results from testing for integration of pPQ126 in different genetic backgrounds and testing for transposition of Tn917 from pTV1ts.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chromosomal target</th>
<th>Selection for</th>
<th>Antibiotic^R cfu 30 C</th>
<th>Antibiotic^R cfu 39 C</th>
<th>Integration frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP1844</td>
<td>none</td>
<td>Em^r</td>
<td>3.20 x 10^9</td>
<td>&lt;1 x 10^4</td>
<td>0</td>
</tr>
<tr>
<td>ISP1844</td>
<td>none</td>
<td>Gm^r</td>
<td>2.60 x 10^9</td>
<td>4.00 x 10^6</td>
<td>0</td>
</tr>
<tr>
<td>JBL85</td>
<td>Tn4001</td>
<td>Em^r</td>
<td>7.60 x 10^8</td>
<td>4.40 x 10^7</td>
<td>5.76 x 10^-2</td>
</tr>
<tr>
<td>ISP1846</td>
<td>Tn551</td>
<td>Em^r</td>
<td>1.40 x 10^10</td>
<td>3.10 x 10^8</td>
<td>2.20 x 10^-2</td>
</tr>
<tr>
<td>ISP1846</td>
<td>Tn551</td>
<td>Gm^r</td>
<td>1.30 x 10^10</td>
<td>4.20 x 10^5</td>
<td>3.30 x 10^-5</td>
</tr>
<tr>
<td>JBL177</td>
<td>Tn4001</td>
<td>Em^r</td>
<td>6.90 x 10^9</td>
<td>8.40 x 10^4</td>
<td>1.20 x 10^-5</td>
</tr>
<tr>
<td>ISP1578</td>
<td>none</td>
<td>Em^r</td>
<td>8.35 x 10^9</td>
<td>2.03 x 10^7</td>
<td>2.43 x 10^-3</td>
</tr>
</tbody>
</table>

^All data were obtained from Tables 14 through 18.

^Transposition frequency of Tn917, not an integration frequency for pTV1ts.
Table 18. Isolation of pPQ126 integration events by transforming ISP5 with DNA extracted from ISP1846 after growth at 43°C with selection.

<table>
<thead>
<tr>
<th>Phenotype (no. scored)</th>
<th>% recombinants in class:</th>
<th>Frequency of Transformation</th>
<th>Reversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Em^R (33)</td>
<td>36.4</td>
<td>21.2</td>
<td>12.1</td>
</tr>
</tbody>
</table>

ISP5 8325 thv-101 thrB106 ilv-129 pig-131
ISP1846 8325-4 pig-131 trp-159::Tn551 ermB317 (pPQ126).

Classes: A, Em^R Gm^R Cm^R (9 ug/ml) Trp^+ Thy^- Thr^-; B, Em^R Gm^S Cm^R (9 ug/ml) Trp^- Thy^- Thr^-; C, Em^R Gm^R Cm^R (9 ug/ml) Trp^- Thy^- Thr^-; D, Em^R Gm^S Cm^R (9 ug/ml) Trp^- Thy^- Thr^-; E, Em^R Gm^R Cm^R (9 ug/ml) Trp^- Thy^+ Thr^-; F, Em^R Gm^R Cm^R (9 ug/ml) Trp^- Thy^- Thr^-; G, Em^R Gm^R Cm^R (9 ug/ml) Trp^+ Thy^+ Thr^-; H, Em^R Gm^R Cm^R (6 ug/ml) Trp^+ Thy^- Thr^-.

Expressed as colonies recovered per 10^9 cfu of the transformation or control suspension spread onto the selective media; 0.66 x 10^9 cfu per ml of the transformation suspension and excess DNA were used. The selective medium was BHI plus 1 ug/ml of erythromycin (Em^R), and all incubations and platings were performed at 39°C.
chromosomal determinants (trp, or tyr, or thr) from ISP1846 to ISP5. To determine whether pPQ126 existed as an autonomous or integrated replicon in these transformants, DNA was extracted from one member of each class. Following digestion with HindIII and agarose-gel analysis, all classes demonstrated the absence of autonomous pPQ126 (data not presented).

The effect of temperature on the stability of chromosomally integrated pPQ126

It was important to confirm that pPQ126 was chromosomally integrated in isolates obtained following growth at elevated temperature in the presence of homologous chromosomal DNA. As described previously for JBL85, it was possible to recover isolates (e.g., JBL113 or JBL114) that displayed the pPQ126 phenotype following growth at 39 °C [Em^Gm^Cm^R(10 ug/ml)]. Furthermore, these isolates were shown by agarose-gel electrophoresis to be lacking autonomous pPQ126. Also recovered, were isolates from 30 °C that contained autonomous pPQ126 [Em^Gm^Cm^R(14 ug/ml)], and isolates from 39 °C that had lost pPQ126 [Em^Gm^Cm^R(6 ug/ml)]. Assuming that pPQ126 was present as an integrated replicon in JBL113 or JBL114, only the elevated temperature (39 °C) was responsible for assuring that pPQ126 was maintained in the integrated state.

To test if lowered temperature would result in excision and autonomous replication of pPQ126, JBL113 was streaked for isolation onto two plates of BHI plus gentamicin (3 ug/ml), erythromycin (3 ug/ml), and chloramphenicol (1 ug/ml); one plate was incubated at 30 °C and the other at 39 °C. A loopful of cells from the 30 °C streak plate was used to inoculate 10 ml of BHI (same selection) for overnight growth at 30 °C, and a loopful of cells
from the 39 C streak plate was used to inoculate 10 ml of BHI (same selection) for overnight growth at 39 C. DNA was extracted from both preparations of JBL113, digested with HindIII, and subjected to agarose-gel electrophoresis (Figure 14). For comparative purposes, DNA was also extracted from JBL85, JBL101, JBL107, JBL108, and ISP1565. Lane 3, the negative control, shows the absence of plasmid DNA in ISP1565. As a positive control, lane 5 contained the 11.1-kb and 2.5-kb HindIII fragments of pPQ126 isolated from JBL85 (grown with selection at 30 C). JBL101 and JBL107 displayed the pPQ126 phenotype at 30 C, and as expected, showed the pPQ126 HindIII pattern in lanes 7 and 9, respectively. JBL108, which is of the pPQ126-minus phenotype, does not harbor pPQ126 DNA (see lane 11). As discussed previously, JBL113 grown at 39 C with selection displayed the phenotype for integrated pPQ126 [Em^Gm^Cm^(10 ug/ml)], and was shown by electrophoretic analysis to lack autonomous pPQ126: lane 15 further supports these earlier results. Following growth of JBL113 at 30 C with selection, however, pPQ126 appeared to excise imprecisely from the chromosome. As shown in lane 13, three HindIII fragments are clearly visible following digestion of DNA extracted from JBL113 grown at 30 C. Two of the bands corresponded exactly to the two HindIII fragments of pPQ126 (compare lane 13 to lane 5). The extra fragment (approximately 7 kb in length) is thought to be chromosomal DNA adjacent to the chromosomal insertion site of pPQ126 (see below).
Figure 14. Analysis of isolates obtained following a high-temperature plasmid-elimination experiment with JBL85

Lane 1, HindIII-digested lambda standard (0.30 ug total); lane 2, uncut ISP1565 DNA (30 C, no selection); lane 3, HindIII-digested ISP1565 DNA (30 C, no selection); lane 4, uncut JBL85 DNA (30 C, gee); lane 5, HindIII-digested JBL85 DNA (30 C, gee); lane 6, uncut JBL101 DNA (30 C, gee); lane 7, HindIII-digested JBL101 DNA (30 C, gee); lane 8, uncut JBL107 DNA (30 C, gee); lane 9, HindIII-digested JBL107 DNA (30 C, gee); lane 10, uncut JBL108 DNA (39 C, no selection); lane 11, HindIII-digested JBL108 DNA (39 C, no selection); lane 12, uncut JBL113 DNA (30 C, gee); lane 13, HindIII-digested JBL113 DNA (30 C, gee); lane 14, uncut JBL113 DNA (39 C, gee); lane 15, HindIII-digested JBL113 DNA (39 C, gee).

All DNAs were extracted via the rapid preparation method from cells grown overnight in 10 ml of BHI broth and incubated at the specified temperature without selection, or in the presence of 3 ug/ml each of gentamicin and erythromycin and 1 ug/ml of chloramphenicol (gee).
Transformation of ISP5 with high molecular weight DNA extracted from JBL85 previously grown at 43°C

High molecular weight DNA was extracted from a population of cells of JBL85 grown at 43°C, and was used to transform competent cells of ISP5. Selection was made for resistance to erythromycin. To determine if pPQ126 would remain integrated following transformation under permissive conditions, all incubations were performed at 34°C. The results of this experiment are presented in Table 19. Although only 7 Em^E transformants were recovered, these transformants comprised three distinct phenotypic classes: class A (3 of 7) were Em^E/Gm^E/Cm^E(12 ug/ml) Ilv^-; class B (2 of 7) were Em^E/Gm^E/Cm^E(12 ug/ml) Ilv^-; and class C (2 of 7) were Em^E/Gm^E/Cm^E(12 ug/ml) Ilv^+. A single member from each class was retained for further analysis: JBL89, class A; JBL88, class B; JBL87, class C. All three classes were resistant to erythromycin and chloramphenicol, and JBL87 and JBL89 were also resistant to gentamicin. It was assumed that some form of autonomous plasmid DNA was present because each isolate was resistant to 12 ug/ml of chloramphenicol. In addition, JBL87 transferred a chromosomal determinant from JBL85 to ISP5 (Ilv^+).

Following extraction and subsequent digestion of DNA isolated from JBL89, JBL88, and JBL87, the DNA preparations were analyzed by agarose-gel electrophoresis (Figure 15). As described below, all 3 isolates harbored at least one fragment of pPQ126 DNA. Lane 7 contains HindIII-cut DNA isolated from ISP5 to show that plasmid DNA was not present prior to transformation with JBL85 DNA. JBL89 DNA (lane 9) contained the 2.5 kb Hind fragment of pPQ126, and 3 additional bands approximately 8.5 kb, 7.5 kb,
Table 19. Isolation and analysis of transformants obtained by transforming ISP5 with DNA extracted from JBL85 after growth at 43 C with selection

<table>
<thead>
<tr>
<th>Phenotype (no. scored)</th>
<th>% recombinants in class:</th>
<th>Frequency of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Em^R (7)</td>
<td>42.86</td>
<td>28.57</td>
</tr>
</tbody>
</table>

^aISP5 8325 thy-101 thrB106 ilv-129 pig-131
JBL85 8325 pig-131 w[Ch::Tn4001]1210 (pPQ126).

^bClasses: A, Em^R Gm^R Cm^R (12 ug/ml) Ilv^-; B, Em^R Gm^R Cm^R (12 ug/ml) Ilv^-; C, Em^R Gm^R Cm^R (12 ug/ml) Ilv^+.

^cExpressed as colonies recovered per 10^9 cfu of the transformation or control suspension spread onto the selective medium; 0.88 x 10^9 cfu per ml of the transformation suspension and excess DNA were used. The selective media was BHI plus 1 ug/ml of erythromycin (Em^R), and all incubations and platings were performed at 34 C.
Figure 15. Analysis of transformants obtained from transformation of ISP5 with DNA extracted from JBL85 previously grown with selection at 43°C.

Lane 1, HindIII-digested lambda standard (0.30 µg total); lane 2, uncut ISP1565 DNA (g); lane 3, HindIII-digested ISP1565 DNA (g); lane 4, uncut JBL85 DNA (gec); lane 5, HindIII-digested JBL85 DNA (gec); lane 6, uncut ISP5 DNA (no selection); lane 7, HindIII-digested ISP5 DNA (no selection); lane 8, uncut JBL89 DNA (gec); lane 9, HindIII-digested JBL89 DNA (gec); lane 10, uncut JBL87 DNA (gec); lane 11, HindIII-digested JBL87 DNA (gec); lane 12, uncut JBL88 DNA (ec); lane 13, HindIII-digested JBL88 DNA (ec); lane 14, HindIII-digested lambda standard (0.30 µg total).

All DNAs were extracted via the rapid preparation method from cells grown overnight in 10 ml of BHI broth and incubated at 34°C without selection, or in the presence of 3 µg/ml of gentamicin (g), or 3 µg/ml of erythromycin and 1 µg/ml of chloramphenicol (ec), or 3 µg/ml each of gentamicin and erythromycin and 1 µg/ml of chloramphenicol (gec).
and 4.7 kb in length; the 11.1 kb fragment of pPQ126 was absent. The plasmid present in JBL89 was designated pLUCH89. DNA extracted from JBL87 (lane 11) contained the 11.1 and 2.5 kb fragments of parental pPQ126. DNA obtained from JBL88 (lane 13) was missing the 2.5 kb HindIII fragment of pPQ126; however, the 11.1 kb HindIII fragment of pPQ126 and an additional 4 fragments approximately 6.5 kb, 2.9 kb, 2.2 kb, and 1.8 kb in size were present. The plasmid present in JBL88 was designated pLUCH88. From Figure 15, it was apparent that absence of the 2.5 kb HindIII fragment was responsible for the Gm^s phenotype of JBL88. The uncut preparation of JBL88 (lane 12) contains a small molecular weight band, that does not harbor a recognition site for HindIII, as evidenced by the presence of a single band at the same position in lanes 13 (HindIII-cut) and 12 (uncut). The relationship of this band to plasmid pPQ126 is unknown.

DNA Hybridizations

For several reasons, most importantly to further confirm that plasmid pPQ126 sequences were chromosomally maintained under non-permissive conditions, it was necessary to perform hybridization analyses on several DNAs. DNA sequences sharing homology with plasmid pPQ126 were cloned into pBR322 for use as hybridization probes. The 4.2-kb HindIII fragment of pI258 (ermB gene of Tn551) and the 2.5-kb HindIII fragment of pPQ58 (aacA-aphD gene of Tn4001) were cloned separately into the unique HindIII site of pBR322 to generate plasmids pLUCH75 (JBL75) and pPQ132 (ISP1872), respectively (John B. Luchansky, Department of Microbiology, Iowa State University, Ames, Iowa, unpublished data). For convenience, ^32[P]-labeled pLUCH75 and ^32[P]-labeled pPQ132 are hereafter referred to as the Em^R and Gm^R probes.
respectively. The molecular weights (length in kilobase pairs) of the majority of the restriction fragments discussed below are estimations based on migration of these fragments compared to PstI- and/or HindIII-digested lambda DNA.

Hybridization of pPQ126 sequences to $^{32}$P-labeled pPQ132 and $^{32}$P-labeled pLUCH75

Plasmid pPQ126 was digested with different restriction endonucleases, and the resulting restriction fragments were fractionated by agarose-gel electrophoresis. The fragments were then transferred from agarose to Magnagraph membranes and hybridized to $^{32}$P-labeled DNA probes (Figure 16). Fragments sharing homology with either pLUCH75 (Em$^R$ probe) or pPQ132 (Gm$^R$ probe) were detected by autoradiography. Allowing for the presence of an additional 2.5-kb HindIII fragment (the Gm$^R$ determinant of Tn4001), the digestion profile of pPQ126 in panel A (Figure 16) compares exactly to the published restriction map of the parental plasmid (pTVl$s$; see Figure 5).

Digestion of pPQ126 with XbaI produced fragments 12.15 kb and 1.45 kb in length (lane 2, panel A), and the 12.15-kb fragment hybridized to both the Gm$^R$ and Em$^R$ probes (lane 2, panels B and C, respectively). Although the 1.45-kb Xba fragment harbors a portion of the erm-distal arm of Tn917, the terminal-repeat segment therein (38 base pairs) did not provide sufficient homology for hybridization (see lane 2, panel C). This result was not surprising, however, because the erm-distal inverted repeat of Tn917 is not entirely homologous to the erm-proximal inverted repeat (Perkins and Youngman, 1984).
Figure 16. Hybridization of $^{32}$P-labeled pPQ132 and $^{32}$P-labeled pLUCH75 to restriction endonuclease-digested pPQ126

Panel A: Agarose gel electrophoresis of plasmid pPQ126 after restriction endonuclease digestion. Lane 1, PstI digest of phage lambda DNA (0.75 ug total); lanes 2 through 9, plasmid pPQ126 digested with XbaI, HindIII, AvaI, XbaI/HindIII, XbaI/AvaI, EcoRI, SalI, and EcoRI/SalI, respectively.

Panel B: Hybridization of $^{32}$P-labeled pPQ132 to pPQ126 DNA, following electrotransfer of the gel in Figure 16A to Magnagraph; lane contents are the same as Figure 16A.

Panel C: Hybridization of $^{32}$P-labeled pLUCH75 to pPQ126 DNA, following electrotransfer of the gel in Figure 16A to Magnagraph; lane contents are the same as Figure 16A.
An 11.1-kb and a 2.5-kb fragment resulted from digestion of pPQ126 with HindIII (lane 3, panel A). As expected, the 11.1-kb fragment (containing Tn917 DNA) hybridized exclusively with the Em^ probe (lane 3, panel C), and the 2.5-kb fragment (containing Tn4001 DNA) hybridized exclusively with the Gm^ probe (lane 3, panel B).

Four fragments (6.45, 4.65, 1.75, and 0.75 kb) were visible following digestion of pPQ126 with AvaI (lane 4, panel A). The 4.65-kb Ava fragment contains segments of pPQ126 homologous with both probes; therefore, this fragment hybridized to both pPQ132 and pLUCH75 (lane 4 of panels B and C, respectively). The 1.75-kb Ava fragment (the erm-proximal arm of Tn912) hybridized exclusively to the pLUCH75 probe (lane 4, panel C).

Lane 5 (panel A) contains pPQ126 DNA digested with both XbaI and HindIII. Three of the expected four fragments are clearly visible (9.42 kb, 2.5 kb, and 1.44 kb). The fourth fragment, approximately 0.24 kb, either exited the gel or was not present in sufficient concentration to be visualized. The 9.42-kb Xba/Hin fragment encompasses the erm gene of Tn917 and hybridized, therefore, to the Em^ probe (lane 5, panel C). The 2.5-kb product (the aacA-anhP gene of Tn4001) of the Xba/Hin double digestion hybridized exclusively to the Gm^ probe (lane 5, panel B).

Lane 6 (panel A) of Figure 16 shows the results for digestion of pPQ126 with both XbaI and AvaI. At least four distinct bands (6.45, 3.94, 1.75, and 0.58 kb in length) are discernible. Based on the restriction map of pPQ126 (see Figure 12), digestion with both XbaI and AvaI would be expected to produce 6 bands, approximately 6.45, 3.94, 1.75, 0.78, 0.58, and 0.10 kb in length. The total concentration of DNA in the 0.10 kb...
fragment was probably below the limits of resolution for this band to be visualized. It is possible that due to incomplete digestion, the 0.78-kb fragment remained associated with a higher-molecular weight fragment, or that reaction conditions promoted further digestion (e.g., star activity) of this fragment to a smaller-molecular weight species. Restriction endonucleases, most notably EcoRI, have been observed to cleave sequences that are similar (though not identical) to their defined recognition sequences. Conditions supporting this star activity include, high endonuclease concentration, low ionic strength, and high concentrations of glycerol or DMSO (Dr. Joan Brooks, New England Biolabs, Beverly, MA, personal communication).

Digestion of pPQ126 with enzymes that cut only once (e.g., EcoRI and SalI) generated a single 13.6-kb fragment that hybridized with both probes [lanes 7 (EcoRI) and 8 (SalI), panels A, B, and C, respectively]. Double digestion of pPQ126 with both EcoRI and SalI yielded the 2 expected fragments, approximately 9.62 kb and 3.98 kb in length (lane 9, panel A). Careful inspection of lane 9 (panel A) shows the presence of an additional band approximately 7.5 kb in length. This band probably arose due to star activity of EcoRI. Although not seen in Figure 16 but discernible in the original autoradiogram, the 7.5-kb "star" fragment demonstrated weak (faint band present) hybridization to the EmF probe (data not shown). The 3.98-kb Eco/Sal fragment, which includes the GmF determinant but not the EmF determinant of pPQ126, hybridized only with the pPQ132 probe (lane 9, panel B). Conversely, the 9.62-kb fragment, which includes the erm gene of
pPQ126 but not the aacA-aphD gene, hybridized only with the pLUCH75 probe (lane 9, panel C).

Hybridization of restriction endonuclease-digested chromosomal DNAs from JBL70, JBL71, and JBL74 to $^{32}$P-labeled pPQ132 and $^{32}$P-labeled pLUCH75 probes to autonomous pPQ126 DNA, these same probes were hybridized to bulk chromosomal DNA which putatively contained integrated pPQ126. CsCl-purified chromosomal DNAs from JBL70, JBL71, and JBL74 (three Em$^R$ isolates obtained following transformation of ISP5 with chromosomal DNA extracted from a population of cells of ISP1846 grown at 43°C; see Table 18) were digested separately with HindIII and with EcoRI. The resulting fragments were transferred to Magnagraph membranes and hybridized to the Em$^R$ and Gm$^R$ probes as described previously. The results of these analyses are presented in Figure 17.

As shown in panel A, digestion of pPQ126 with HindIII (lane 1) produced fragments 11.1 kb and 2.5 kb in length, and digestion of this plasmid with EcoRI (lane 5) produced a single 13.6-kb fragment. As expected, the 11.1-kb Hin fragment hybridized only to the Em$^R$ probe (panel C, lane 1), and the 2.5-kb Hin fragment hybridized only to the Gm$^R$ probe (panel B, lane 1). Lane 5 of panels B and C demonstrated hybridization of both probes to the single 13.6-kb EcoRI fragment of pPQ126.

Digestion of CsCl-purified DNAs from JBL70, JBL71, and JBL74 with either HindIII (panel A, lanes 2, 3, and 4) or EcoRI (panel A, lanes 6, 7, and 8) generated the expected ladder of chromosomal fragments. Hybridization of EcoRI-digested DNA from the transformants to the Em$^R$ probe
Figure 17. Hybridization of $^{32}$P-labeled pPQ132 and $^{32}$P-labeled pLUCH75 to restriction endonuclease-digested total DNA from JBL70, JBL71, and JBL74, and plasmid pPQ126

Panel A: Agarose gel electrophoresis of chromosomal DNA isolated from JBL70, JBL71, and JBL74, and plasmid pPQ126 DNA, after restriction endonuclease digestion. Lanes 1 through 4, HindIII digests of: lane 1, pPQ126; lane 2, JBL70; lane 3, JBL71; lane 4, JBL74. Lanes 5 through 8, EcoRI digests of: lane 5, pPQ126; lane 6, JBL70; lane 7, JBL71; lane 8, JBL74.

Panel B: Hybridization of $^{32}$P-labeled pPQ132 to the DNA restriction fragments shown in Figure 17A, following electrotransfer of this gel to Magnagraph; lane contents are the same as Figure 17A.

Panel C: Hybridization of $^{32}$P-labeled pLUCH75 to the DNA restriction fragments shown in Figure 17A, following electrotransfer of this gel to Magnagraph; lane contents are the same as Figure 17A.
(panel C, lanes 6, 7, and 8) yielded a single high-molecular weight (greater than 15 kb) band for each isolate. The band for EcoRI-digested JBL74 that hybridized to the Em probe (panel C, lane 8) was of lower molecular weight than the bands for JBL70 and JBL71 (panel C, lanes 6 and 7, respectively). Hybridization of HindIII-digested DNAs from these transformants to the Em probe (panel C, lanes 2, 3, and 4) generated a band approximately 4.65 kb for JBL70 and JBL71 (panel C, lanes 2 and 3, respectively), and a band approximately 3.5 kb for JBL74 (panel C, lane 4).

Hybridization of HindIII- and EcoRI-digested JBL70, JBL71, and JBL74 DNAs to the Gm probe demonstrated the absence of homology (no hybridization signal) for JBL70 (panel B, lanes 2 and 6, respectively), and a different EcoRI pattern for JBL71 compared to JBL74 (panel B, lanes 7 and 8, respectively). The Gm phenotype of JBL70 probably resulted from deletion of the Gm portion of pPQ126 during integration and/or transformation. The band for EcoRI-digested JBL74 that hybridized to the Gm probe (panel B, lane 8) was of lower molecular weight than the corresponding band for JBL71 (panel B, lane 7).

Hybridization of HindIII-digested pLUCH88 and pLUCH89 DNA to $^{32}$P-labeled pPQ132 and $^{32}$P-labeled pLUCH75

To further clarify the derivation of the additional fragments present in the Em isolates obtained by transforming competent cells of ISP5 with DNA extracted from a population of cells of JBL65 grown at 43 (see Table 19), the Em and Gm probes were hybridized to pLUCH88 and pLUCH89 DNA. As shown in panel A of Figure 18, HindIII-digested DNAs from ISP565 (lane 2) and ISP5 (lane 3) produced a ladder of bands, and pPQ126 (lane 4) generated
Figure 18. Hybridization of $^{32}$P-labeled pPQ132 and $^{32}$P-labeled pLUCH75 to HindIII digests of plasmids pPQ126, pLUCH88, and pLUCH89, and total DNA from ISP1565 and ISP5.

Panel A: Agarose gel electrophoresis of plasmid and total DNA digested with the restriction endonuclease HindIII. Lane 1, PstI digest of phage lambda DNA (0.75 ug total); lane 2, ISP1565; lane 3, ISP5; lane 4, pPQ126; lane 5, pLUCH88; lane 6, pLUCH89.

Panel B: Hybridization of $^{32}$P-labeled pPQ132 to the DNA restriction fragments shown in Figure 18A, following electrotransfer this gel to Magnagraph; lane contents are the same as Figure 18A.

Panel C: Hybridization of $^{32}$P-labeled pLUCH75 DNA to the DNA restriction fragments shown in Figure 18A, following electrotransfer this gel to Magnagraph; lane contents are the same as Figure 18A.
the 11.1-kb and 2.5-kb fragments. The Gm<sup>R</sup> probe (panel B, lane 2) hybridized to a 2.5-kb fragment of HindIII-digested ISP1565 DNA [a fragment derived from the chromosomal insertion of Tn<sub>4001</sub> (w[Chr::Tn<sub>4001</sub>1210]) in ISP1565]. The Em<sup>R</sup> probe did not hybridize to ISP1565 (panel C, lane 2). As expected, ISP5 DNA did not hybridize to either probe (panels B and C, lane 3). For HindIII-digested pPQ126 DNA, the 11.1-kb fragment hybridized to the Em<sup>R</sup> probe (panel C, lane 4), and the 2.5-kb fragment hybridized to the Gm<sup>R</sup> probe (panel B, lane 4).

Digestion of pLUCH88 with HindIII produced 5 distinct bands: 11.1 kb, 6.5 kb, 2.9 kb, 2.2 kb, and 1.8 kb (panel A, lane 5). The 11.1-kb fragment hybridized to the Em<sup>R</sup> probe (panel C, lane 5), and the two smallest fragments (2.2 kb and 1.8 kb) hybridized to the Gm<sup>R</sup> probe (panel B, lane 5). The 6.5- and 2.9-kb fragments of HindIII-digested pLUCH88 did not hybridize to either of the probes tested (panels B and C, lane 5).

Digestion of pLUCH89 with HindIII generated 4 distinct fragments, approximately 8.5, 7.5, 4.7, and 2.5 kb in length (panel A, lane 6). The two smaller fragments (4.7 kb and 2.5 kb) hybridized to the Gm<sup>R</sup> probe (panel B, lane 6), and the two larger fragments (8.5 kb and 7.5 kb) hybridized to the Em<sup>R</sup> probe (panel C, lane 6).

Hybridization of ISP5, pPQ126, and pLUCH88 DNAs to <sup>32</sup>P-labeled pLUCH88

To determine the origin of the additional fragments present in pLUCH88 (JBL88), CsCl-purified plasmid pLUCH88 DNA was labeled with <sup>32</sup>P-dCTP and probed to chromosomal DNA from ISP5 and plasmids pPQ126 and pLUCH88 (Figure 19). Uncut pPQ126 (panel A, lane 2) contained a dominant CCC band, and trace amounts of linear, OC, and multimeric configurations. Digestion of
Figure 19. Hybridization of $^{32}$P-labeled pLUCH88 to uncut and HindIII-digested preparations of total DNA from ISP5 and plasmids pPQ126 and pLUCH88

Panel A: Agarose gel electrophoresis of plasmids pPQ126 and pLUCH88 and chromosomal DNA from ISP5. Lane 1, HindIII digest of phage lambda DNA (0.75 µg total). Lanes 2 through 4, uncut preparations of: lane 2, pPQ126; lane 3, ISP5; lane 4, pLUCH88. Lanes 5 through 7, HindIII digests of: lane 5, pPQ126; lane 6, ISP5; lane 7, pLUCH88.

Panel B: Hybridization of $^{32}$P-labeled pLUCH88 to the gel in Figure 19A, following electrotransfer of this gel to Magnagraph; lane contents are the same as Figure 19A.
pPQ126 with HindIII (panel A, lane 5) generated the 11.1-kb and 2.5-kb fragments.

Several plasmid bands were present in the uncut preparation of pLUCH88 DNA (panel A, lane 4). Although the origin of these additional plasmid species remains unknown, it was determined that pLUCH88 bands (panel A, lane 4) that migrated faster than (below) the 6.6 kb fragment of HindIII-digested lambda DNA did not hybridize to the Em\textsuperscript{r} or Gm\textsuperscript{r} probes (data not presented).

As expected, all sequences of uncut and HindIII-digested pPQ126 and pLUCH88 DNAs hybridized to the \textsuperscript{32}P-labeled pLUCH88 probe (panel B, lanes 2 and 5 and lanes 4 and 7, respectively). Identical results were obtained when \textsuperscript{32}P-labeled pPQ126 was utilized to probe the gel in Figure 19A (data not shown).

For ISP5 DNA, a single, high-molecular weight band for the uncut preparation and a ladder of bands for the HindIII-digested preparation were observed (panel A, lanes 3 and 6, respectively). The pLUCH88 probe did not hybridize with ISP5 DNA. These results suggest that plasmid DNA(s) of JBL88 were not of chromosomal origin; therefore, these sequences are most probably derived from pPQ126, or deleted derivatives of plasmids pPQ126 and/or pLUCH88. As a control, plasmids pPQ126, pLUCH75, and pPQ132 were separately labeled with \textsuperscript{32}P-dCTP and separately hybridized to the gel in Figure 19A; these probes did not hybridize to ISP5 DNA (data not presented).
Hybridization of ISP5, ISP1565, pLUCH113, and pPQ126 DNAs to $^{32}\text{P}$-labeled pPQ126 and $^{32}\text{P}$-labeled pLUCH113

Plasmid pLUCH113 was generated by growing JBL113 (contains chromosomally integrated pPQ126 when maintained at 39 °C) at a permissive temperature (30 °C) in the presence of 3 µg/ml each of erythromycin and gentamicin and 1 µg/ml of chloramphenicol (see Figure 14). To determine the origin of the additional 7.0-kb fragment of HindIII-digested pLUCH113, CsCl-purified pLUCH113 and pPQ126 DNAs were labeled with $^{32}\text{P}$-dCTP and hybridized to total DNA from ISP5 and ISP1565 and to plasmids pPQ126 and pLUCH113 DNAs. As shown in Figure 20A, uncut total DNA from ISP5 and ISP1565 yielded a dense, high-molecular weight band (panel A, lanes 2 and 3, respectively). These same DNAs produced the expected ladder of fragments when digested with HindIII (panel A, lanes 6 and 7, respectively). Uncut pLUCH113 (panel A, lane 4) displayed a dominant CCC band that migrated to the same relative position as the CCC band of uncut pPQ126 DNA (panel A, lane 5). The dominant CCC band for both pLUCH113 and pPQ126 occupied the same position in Figure 20A (lanes 4 and 5, respectively) as the 9.5-kb fragment of the HindIII-digested lambda standard (panel A, lane 1). Also present in the uncut preparation of pLUCH113 was linear, OC, and multimeric configurations; however, there was also an additional (higher molecular weight) CCC band. The uncut preparation of pPQ126 (panel A, lane 5) showed traces of linear and OC conformations, but no additional CCC band. As shown in lane 8 (Figure 20A), digestion of pLUCH113 DNA with HindIII yielded the 11.1-kb and 2.5-kb fragments, as well as an additional fragment approximately 7.0 kb in size.
Figure 20. Hybridization of $^{32}$P-labeled pPQ126 and $^{32}$P-labeled pLUCH113 to uncut and HindIII-digested preparations of total DNA from ISP5 and ISP1565 and plasmid pLUCH113, and uncut plasmid pPQ126 DNA

Panel A: Agarose gel electrophoresis of chromosomal DNA from ISP5 and ISP1565 and plasmids pPQ126 and pLUCH113. Lane 1, HindIII digest of phage lambda DNA (0.9 ug total). Lanes 2 through 5, uncut preparations of: lane 2, ISP5; lane 3, ISP1565; lane 4, pLUCH113; lane 5, pPQ126. Lanes 6 through 8, HindIII digests of: lane 6, ISP5; lane 7, ISP1565; lane 8, pLUCH113.

Panel B: Hybridization of $^{32}$P-labeled pPQ126 to the gel in Figure 20A, following electrotransfer of this gel to Magnagraph; lane contents are the same as Figure 20A.

Panel C: Hybridization of $^{32}$P-labeled pLUCH113 to the gel in Figure 20A, following electrotransfer of this gel to Magnagraph; lane contents are the same as Figure 20A.
Both probes demonstrated weak hybridization (faint signal) to non-digested ISP5 DNA (panels B and C, lane 2). As shown in Table 1, ISP5 does not contain chromosomal insertions of either Tn551 or Tn4001 or sequences that may share homology with pPQ126; therefore, the signal generated in lane 2 of panels B and C was not expected. HindIII-digested ISP5 DNA did not hybridize to $^{32}\text{P}$-labeled pPQ126. As a consequence of digestion with HindIII, it is possible that sequences responsible for hybridization of pPQ126 to uncut ISP5 DNA were distributed throughout the ladder of fragments and, therefore, were not present in sufficient length and/or concentration to generate a hybridization signal. The faint signal in lane 6 of panel C demonstrated that the pLUCH113 probe hybridized to a high molecular-weight fragment (greater than 15 kb) of HindIII-digested ISP5 DNA. This fragment was more apparent after longer exposure of Magnagraph (containing sequences present in Figure 20A) to additional X-ray films (data not presented). Because $^{32}\text{P}$-labeled pLUCH113 DNA (but not $^{32}\text{P}$-labeled pPQ126 DNA) hybridized to HindIII-digested chromosomal DNA from ISP5, it was concluded that the hybridization signal (panel C, lane 6) was mediated by the additional 7.0-kb fragment present in pLUCH113.

Uncut DNA from ISP1565 hybridized to both probes (panels B and C, lane 3) due to the presence of a chromosomal insertion of Tn4001 in this strain. A 2.5-kb fragment (that included the aacA-aphD gene of Tn4001) was expected among the ladder of fragments generated following digestion of total DNA from ISP1565 with HindIII. Both probes, therefore, hybridized to a 2.5-kb fragment of HindIII-digested ISP1565 DNA (panels B and C, lane 7). Furthermore, the pLUCH113 probe hybridized to 2 additional fragments of
HindIII-digested ISP1565 DNA (panel C, lane 7). The larger of the two fragments (about 15 kb) occupied the same relative position (migrated the same distance) as the fragment of HindIII-digested ISP5 DNA that also hybridized to $^{32}\text{P}$-labeled pLUCH113. The smaller of the two fragments (about 8.0 kb) was unique to ISP1565 DNA.

Digestion of pLUCH113 DNA with HindIII yielded 3 distinct fragments (11.1, 7.0, and 2.5 kb). Although both probes hybridized to the 11.1- and 2.5-kb fragments, only pLUCH113 hybridized to the additional 7.0-kb fragment (compare panels B and C, lane 8). Based on this data, it was concluded that the additional 7.0-kb fragment present in pLUCH113 was not derived from plasmid pPQ126.

As a consequence of temperature-downshift [from non-permissive (39°C) to permissive (30°C) conditions] in the presence of selective levels of erythromycin, gentamicin, and chloramphenicol, plasmid pPQ126 and chromosomal DNA were excised from the chromosome (Figures 14 and 20). This process is hereafter referred to as plasmid disintegration.
DISCUSSION

Several investigators have utilized integrable plasmids to facilitate genomic analysis and gene cloning in *B. subtilis* (Fahnestock et al., 1986; Ferrari et al., 1983; Niaudet et al., 1982; Young, 1983). Plasmid insertion vectors have been useful for chromosomal mapping (Haldenwang et al., 1980), for cloning and studying genes involved in sporulation (Youngman et al., 1984a), for gene replacement studies (Stahl and Ferrari, 1984), and for complementation analysis (Saunders et al., 1984a). These plasmids are inherited by recombination between plasmid sequences and homologous DNA present on the chromosome of a Gram-positive host. The primary objective of this study was to develop an integrable plasmid to facilitate the genomic analysis of *S. aureus*.

Initially, efforts were directed to adapt an established insertion vector for use in *S. aureus*. An approach similar in principle to that of Youngman et al. (1984a) for constructing vectors to recover chromosomal Tn917 insertions was employed in this study. The utility of the insertion vector (pLUCH3) was increased by using a portion of a transposon (the *ermB* gene of Tn551) as the homologous segment. Integration could theoretically be directed into any region of the chromosome containing a copy of the same (Tn551) or a similar transposon (Tn917). Several insertions of Tn551 were available for targeting plasmid integration (Luchansky and Pattee, 1984), and several chromosomal insertions of Tn917 have recently been obtained in *S. aureus* (J. B. Luchansky, Department of Microbiology, Iowa State University, Ames, Iowa, unpublished data; C. Hasegawa, Department of Microbiology, Iowa State University, Ames, Iowa, personal communication).
Several genes from plasmid pI258 have been cloned, including \textit{blaZ} (Saunders et al., 1984a), and the genes encoding for resistance to mercury (\textit{merA} and \textit{merB}) and cadmium ([(\textit{cadA} and \textit{cadB}); Witte et al., 1986]). Prior to cloning the \textit{ermB} gene of Tn551, however, it was necessary to confirm the presence of various restriction sites on plasmids pJH101 and pI258. The results in Figure 6 after digestion of these two plasmids with EcoRI, and digestion of pJH101 with HindIII, compare well with published results (Novick et al., 1979c; Ferrari et al., 1983). The only anomaly was the presence of 13 distinct HindIII fragments of pI258, compared to the eleven HindIII fragments reported by Wilson and Baldwin (1978). For the purposes of this study, it was only necessary to identify and recover the HindIII fragment of pI258 (\textit{ermB} gene). It should also be mentioned that it was not possible to enrich for pJH101 with either spectinomycin (300 \text{ug/ml}) or chloramphenicol (170 \text{ug/ml}). There was no discernible difference in DNA concentration between amplified and non-amplified preparations of pJH101 as determined by spectrophotometric and electrophoretic analysis (data not shown). These results were not expected because pJH101 replicates in \textit{E. coli} by using an amplifiable Col El-derived origin of replication (Clewell, 1972). It is possible that the lower than expected yield of plasmid DNA from amplified preparations reflected a decreased source of DNA (fewer cells in the amplified compared to the non-amplified preparation because of bacteriostatic effects of these antibiotics), and not the inability to amplify for pJH101. Efforts were not directed to examine the amplification process in more detail.
The 2.8-md HinB fragment of plasmid pI258 was isolated directly from agarose and ligated to CIP-treated, HindIII-digested pJH101 to generate plasmid pLUCH3. Surprisingly, few E. coli transformants were obtained (data not shown). The procedure utilized to transform competent cells of E. coli was reported by Dagert and Ehrlich (1979) to routinely yield $2 \times 10^7$ transformants per ug of pBR322 DNA. This value does not compare favorably with the $4.5 \times 10^3$ transformants recovered per ug of pLUCH3 DNA (data not presented). At least two explanations can be proposed to explain this anomaly. As a consequence of CIP-treatment, one nick would remain unligated at each join of pLUCH3, and nicked or open circular molecules transform E. coli less efficiently than CCC molecules (Cohen et al., 1973). Also, plasmid pLUCH3 (6.39 md) is larger than pBR322 (2.9 md), and the transformation frequency decreases as the size of the plasmid increases (Hanahan, 1983). Of the $10^5$ Ap$^\square$ transformants obtained, about 7% (7 of 105) were also Tc$^\square$, and only about 2% (2 of 105) contained S. aureus passenger DNA (data not shown). Lee and Ianolo (1985) reported that approximately 75% of the E. coli transformants recovered when cloning S. aureus passenger DNA into the BamHI site of CIP-treated pBR322 were putative recombinant-containing clones. Ferrari et al. (1983), however, reported that only 27% of the Ap$^\square$ transformants were Tc$^\square$ when B. subtilis passenger DNA was cloned into the BamHI site of a derivative of plasmid pBR322. Because the HindIII site of pJH101 lies within the Pribnow box of the Tc$^\square$ gene, many false-negative (Tc$^\square$) transformants could have resulted from the initiation of transcription of the Tc$^\square$ gene from a promoter on the passenger fragment, or by re-creation of the site upon insertion of
passenger DNA. The lowered transformation frequency, as well as the poor recovery of recombinant molecules among the transformants that were obtained, could be accounted for by sub-optimal conditions for transformation, ligation, and CIP-treatment. Although the desired recombinant plasmid was obtained (pLUCH3), future efforts should be directed to optimize conditions for improved recovery of transformants containing recombinant plasmids.

It is noteworthy that the \textit{ermB} gene of Tn551 was expressed from plasmid pLUCH3 in \textit{E. coli}. Of the four erythromycin-resistant determinants available in \textit{S. aureus}, only the \textit{ermA} [(Tn554); Murphy and Lofdahl, 1984] and \textit{ermC} [(pE194); Barany et al., 1982] genes have been reported previously to be expressed in \textit{E. coli}. The \textit{erm} determinant of Tn917 does not express at selectable levels in \textit{E. coli} (Youngman et al., 1984a). Despite extensive DNA sequence homology and restriction map similarity (Shaw and Clewell, 1985; Perkins and Youngman, 1984), Tn551 and Tn917 encode for constitutive and inducible resistance to erythromycin, respectively, in \textit{S. aureus}. The same factors responsible for the regulation of these genes in \textit{S. aureus} (e.g., different base sequences of the promoter regions) may also allow for the recognition and expression of only the \textit{ermB} gene of Tn551 in \textit{E. coli}.

Genomic analysis of \textit{S. aureus} has suffered from the absence of a well-defined, high-efficiency system for introducing DNA into competent cells. Although comparisons are often made with other Gram-positive systems (e.g., \textit{B. subtilis}), very little is actually known concerning the specific mechanism(s) that \textit{S. aureus} utilizes for binding, internalizing,
and processing DNA. Several investigators have transformed competent cells of \textit{S. aureus} with chromosomal or plasmid DNA (Lindberg and Novick, 1973; Lindberg et al., 1972; Lofdahl et al., 1978a; Luchansky and Pattee, 1984; Pattee and Neveln, 1975), but it has not been possible to routinely transform plasmid DNA into \textit{S. aureus} in our laboratory (C. D. Bortner and J. B. Luchansky, Department of Microbiology, Iowa State University, Ames, Iowa, unpublished data). A few transformants were recovered by using plasmid pCl94; however, transformants were not obtained with plasmids pLUCH3 or pI258, despite the fact that ISP130 was competent, as evidenced by the recovery of Em\textsuperscript{r} transformants when chromosomal DNA was used (Table 4). Lofdahl et al. (1978a) obtained transformants of competent \textit{S. aureus} at frequencies ranging from $10^2$ to $10^3$ per ug of OG or CCC DNA; linear DNA produced transformants at 10-fold lower frequency.

Efforts were made to modify the transformation protocol to more closely approximate the conditions used by Lofdahl et al. (1978a), but these alterations did not result in improved recovery of plasmid-containing transformants (data not shown). Attempts to introduce linear and CCC plasmid molecules into competent cells that contained a homologous plasmid (e.g., marker-rescue transformation), were also unsuccessful (C. D. Bortner, Department of Microbiology, Iowa State University, Ames, Iowa, personal communication; J. B. Luchansky, Department of Microbiology, Iowa State University, Ames, Iowa, unpublished data). It is likely that several factors [e.g., DNA purity, the presence of extracellular nuclease(s), or characteristics of the recipient strain] contributed to low recoveries of transformants in this study. Although the plasmid DNA preparations used
for transformations were not purified by CsCl centrifugation, other investigators have obtained transformants by using bulk DNA isolated from plasmid-containing \textit{S. aureus} donor strains (Lindberg and Novick, 1973). Most strains of \textit{S. aureus} produce an extracellular DNase and various restriction enzymes, any of which could degrade exogenously supplied DNA. A DNase-minus, restrictionless strain of \textit{S. aureus} has been constructed to circumvent this problem (Kondo and Yoshizawa, 1985). Sjostrom et al. (1979) also obtained better recovery of plasmid DNA by using a restriction-deficient recipient strain of \textit{S. aureus} for competent-cell transformation.

After repeated failures to introduce pLUCH3 into \textit{S. aureus} via competent-cell transformation, efforts were directed toward the transformation of protoplasts with plasmid DNA. Lindberg (1981) obtained a 10-fold increase in the total number of plasmid-containing transformants for protoplast transformation compared to competent-cell transformation of \textit{S. aureus}; however, protoplasts of \textit{S. aureus} are not transformed with chromosomal or linear DNA (Lindberg, 1981; O'Reilly et al., 1986). A protoplast transformation system based on the Chang and Cohen (1979) protocol for transforming protoplasts of \textit{B. subtilis} with plasmid DNA was adapted for use in this study. As shown in Table 5, pG194 and pI258 transformed protoplasts of ISP130 at frequencies of 1.18 x 10^4 and 8.6 x 10^0, respectively. When competent-cell transformation of ISP130 (Table 4) is compared with protoplast transformation of ISP130 (Table 5), it is obvious that protoplasts of \textit{S. aureus} take up plasmid DNA more readily than whole cells take up plasmid DNA. It was not possible, however, to
introduce the insertion vector pLUC3 into *S. aureus* by either procedure. Nevertheless, based on the encouraging results of some preliminary experiments, I decided to optimize the protoplast transformation protocol to enhance the uptake of plasmid DNA by *S. aureus*.

Two critical parameters of the protoplast transformation protocol were the plating regimen and the medium employed to plate the protoplast-DNA mixture. As shown in Table 6, R agar routinely yielded more transformants than DM3 agar. More pC194 transformants were recovered on R agar containing chloramphenicol supplied in an agar overlay than R agar with chloramphenicol directly incorporated into the medium. When DM3 agar was used, however, more pC194 transformants were recovered when chloramphenicol was incorporated directly into the medium than when it was omitted. Chang and Cohen (1979) selected for pCl94 transformants on DM3 agar containing chloramphenicol, but Lindberg (1981) employed an agar overlay selection when adapting this protocol to *S. aureus*, presumably because it produced higher frequencies. For transformation of *Streptococcus lactis* protoplasts with plasmid DNA, Kondo and McKay (1984) determined that agar overlays stimulated recovery of transformants (possibly by enhancing the regeneration frequency), and negated the variability in results obtained when spread plates were used. Regardless of the regeneration medium or the manner in which the selective agent was applied, more transformants were recovered when the protoplast-DNA mixture was allowed to incubate at 30°C for 3.5 h prior to plating (plating method 2), than when this mixture was plated immediately (plating method 1). Based on the data in Table 5 (and because R agar without an overlay was considerably less time-consuming and
less complicated to prepare), the protoplast-DNA mixture was plated on R agar plus the selective agent after a 3.5-h incubation at 30 C to enable phenotypic expression of plasmid-borne determinants. It was also determined that R agar containing 10 ug/ml of chloramphenicol significantly reduced the amount of background growth and yielded more transformants than R agar containing 3 ug per ml of chloramphenicol. Protoplasts require a solid support to initiate cell wall synthesis, and regenerating protoplasts are inhibited ("crowded out") by the presence of early-forming colonies (Chen et al., 1986; Stahl, 1982; Stahl and Pattee, 1983a). Furthermore, chloramphenicol (5 ug/ml) inhibits regeneration of S. aureus protoplasts (Gruss and Novick, 1986). Elimination of non-protoplasted units and plasmid-free protoplasts precluded the "crowding out" of protoplasts that could tolerate elevated levels of chloramphenicol (bona fide pC194-containing protoplasts).

CsCl-purified preparations of pC194 and pTV1r yielded significantly more transformants than crude preparations (Table 7). CsCl equilibrium density centrifugation removes contaminating chromosomal DNA that would compete with plasmid DNA for cell-surface DNA binding sites, and contaminating nucleases that would nick CCC molecules. Dialysis of plasmid DNA to remove cesium chloride also removes residual SLS, and protoplasts are highly sensitive to even trace amounts of detergent. Any of the above consequences of CsCl purification could account for the enhanced recovery of protoplast plasmid transformants when using CsCl-purified DNA. As shown in Table 7, more transformants were recovered following induction for gene expression, and for a small plasmid (pC194) compared to a large plasmid
For transformation of protoplasts of *S. carnosus*, the number of transformants obtained per ug of pC194 (2.9 kb) was at least two orders of magnitude higher than the number of transformants obtained with pI258 [(28.2 kb); Gotz et al., 1983]. Smaller plasmids also transformed protoplasts of *Streptococcus lactis* at significantly higher frequencies than larger plasmids (Kondo and McKay, 1984).

Heat treatment of recipient cells prior to the addition of phage lysates (Asheshov and Jevons, 1963) or plasmid DNAs (Sjostrom et al., 1979) resulted in higher transfer frequencies, probably because of thermal inactivation of restriction/modification enzymes. When the data in Table 8 for plasmids pTV20 (isolated from *B. subtilis*) and pTV1ts (isolated from *S. aureus*) are compared, it is evident that the origin of the DNA largely determines if protoplasts of *S. aureus* can be transformed with a particular plasmid. Heat treatment of protoplasts prior to the addition of plasmid DNA increased the efficiency of heterologous plasmid transformation (for pTV20 isolated from *B. subtilis*), and increased the frequency at which large plasmids were internalized (pI258).

Depending on the recipient strain and the conditions of the experiment, pC194-containing transformants were recovered at frequencies as high as $3.08 \times 10^5$ (ISP1047; Table 7). This value is considerably higher than frequencies previously reported from this laboratory [(10² to 10³ transformants per ug of CsCl-purified pC194); Jones, 1985], and slightly higher than the $2.6 \times 10^5$ transformants per ug of pC194 DNA reported by Lindberg (1981). Plasmid pC194 transforms protoplasts of *B. subtilis* at a frequency of $1.8 \times 10^7$ per ug of DNA (Chang and Cohen, 1979). The higher
frequency of transformation for *B. subtilis* could be accounted for by the higher regeneration frequency exhibited by protoplasts of *B. subtilis* (50%) compared to protoplasts of *S. aureus* [(10 to 22%); Gruss and Novick, 1986; Stahl, 1982; Stahl and Pattee, 1983a].

After defining the optimal conditions for protoplast transformation, several experiments were repeated in attempts to introduce pLUCH3 into various protoplasted recipient strains of *S. aureus*; however, these were not successful. Any or all of the following may have contributed to the failure to introduce and/or establish pLUCH3 in *S. aureus*: digestion by extracellular and/or intracellular nucleases; low efficiency of transformation; and insufficient production of chloramphenicol acetyl transferase due to a single copy (chromosomally integrated pLUCH3) of the *cat* gene. To assure that vector DNA was established in an appropriate recipient strain prior to insisting that this DNA integrate into the chromosome, a temperature-sensitive insertion vector (based on plasmid pTV1<sub>ts</sub>) was constructed. In preliminary experiments, a severe reduction in recovery of pTV1<sub>ts</sub> transformants was observed when selecting on R agar plus chloramphenicol compared to erythromycin (Table 9). Upon further analysis, the transformants recovered from the chloramphenicol selection plates were found to comprise three distinct phenotypic classes, and two of these classes harbored deleted derivatives of pTV1<sub>ts</sub> (Table 10 and Figure 8). Assuming that only segments involved in plasmid maintenance, replication, and incompatibility must be conserved to avoid hereditary instability, all other segments of a plasmid are subject to genetic rearrangements. Repetitive sequences, transposons, and site-specific recombination systems
all have been implicated in causing deletions (Kleckner, 1981; Murphy and Novick, 1980; Novick et al., 1980; Novick et al., 1984). Given the precedence for such rearrangements, it seems plausible that Tn917 sequences mediated deletions of pTV1-ts when selection was made for resistance to chloramphenicol. The fact that both JBL10 and JBL11 (classes I and II, respectively) were EmR further supports the hypothesis that Tn917 was involved. Deletions were not observed when selection was made for resistance to erythromycin, because these conditions insist for retention of Tn917 sequences. The decreased transformation frequency for chloramphenicol selection can be attributed to deletions in pTV1-ts that were lethal. Deleted derivatives of pTV1-ts (pLUCH10 and pLUCH11) were resistant to higher levels of chloramphenicol than the parental plasmid. In S. aureus, pC194 (a 2.9 kb plasmid present at 40 copies per cell in S. aureus) encodes for resistance to 50 μg/ml of chloramphenicol. The cat gene from pC194 when present on the 12.4 kb plasmid pTV1-ts (copy number at 30 C of 6 per cell in B. subtilis) encodes for resistance to about 12 to 15 μg/ml of chloramphenicol in S. aureus. It follows that the smaller size and higher copy number of plasmids pLUCH10 and pLUCH11 resulted in elevated levels of resistance to chloramphenicol. In subsequent experiments, R agar plus erythromycin was used to select for pTV1-ts (or derivatives of pTV1-ts) to avoid the loss of plasmid material via deletion.

The basic premise was to direct the insertion of pTV1-ts into the chromosome under non-permissive conditions via homologous recombination between plasmid-borne Tn917 and a specific chromosomal copy of Tn551. To adapt pTV1-ts for use as an insertion vector, however, it was necessary to
inactivate the transposition functions of Tn917 (to facilitate the isolation and analysis of plasmid insertion events apart from transposition events), and to add a readily selectable marker (to permit positive selection of cells containing an integrated plasmid). A different selectable marker was required for two reasons: 1) chloramphenicol selection resulted in decreased recovery and deletions of pTV1<sub>CS</sub>; and 2) most recipient strains would already be resistant to erythromycin because of the presence of the chromosomal Tn551 target. It was possible to both add a selectable marker to pTV1<sub>CS</sub> and to inactivate the transposition functions of Tn917. This was accomplished by removing the 1.3-kb HindIII fragment of pTV1<sub>CS</sub> and replacing it with the 2.5-kb gentamicin-resistance HindIII fragment from pPQ58. As described previously, a ligation mixture of HindIII-digested pPQ58 and HindIII-digested, CIP-treated pTV1<sub>CS</sub> was presented to protoplasts of ISP1658 to generate the temperature-sensitive insertion vector pPQ126. The use of a restriction-deficient recipient strain (ISP1658), the selection for Em<sup>R</sup> and not Cm<sup>R</sup> transformants, and the improved protoplast transformation system, all were critical for recovery of pPQ126. Based on the results shown in Figure 11, pPQ126 is a 13.6-kb plasmid composed of an 11.1-kb HindIII fragment (containing replication functions and genes encoding for chloramphenicol and erythromycin resistance from plasmid pTV1<sub>CS</sub>), and a 2.5-kb HindIII fragment (containing the gene encoding for gentamicin resistance from Tn4001 on plasmid pPQ58). The gentamicin-resistance determinant can also be used to direct the integration of pPQ126 into a chromosomal copy of Tn4001.
Table 11 lists the transposition frequencies for Tn4001, Tn551, and Tn917 from various temperature-sensitive delivery vehicles. The transposition frequency of Tn917 (from pTV1) in B. subtilis was estimated at $5 \times 10^{-5}$ to $5 \times 10^{-4}$ (Youngman, 1985). Frequencies of $1.46 \times 10^{-3}$ (ISP1483; Table 11) and $2.43 \times 10^{-3}$ (ISP1578; Table 16) were recorded for transposition of Tn917 from pTV1 ts in S. aureus. The increase in transposition frequency for Tn917 in S. aureus could be accounted for if a transposition event occurred by chance early (e.g., lag phase), and subsequent growth allowed the insertion to attain an abnormally high level among the total population. It should be mentioned, however, that transposition of Tn917 from pTV1 in B. megaterium occurs at frequencies as high as $1.5 \times 10^{-2}$ (Bohall and Vary, 1986). For the purposes of this study, it was only necessary to confirm that the altered derivatives of Tn917 and Tn4001 present on pPQ126 were transpositionally defective. When pPQ126-containing cells (ISP1844) were plated on BHI plus erythromycin and incubated at 39 C, transposition of Tn917 was not observed (Table 11). Similarly, when cells of strain ISP1844 were plated on BHI plus gentamicin, it was not possible to detect transposition events of the 2.5-kb HindIII fragment of Tn4001. The transposition experiments with ISP1844 also demonstrated that pPQ126 harbored a temperature-sensitive replication defect. When cells of strain ISP1844 were plated on erythromycin or gentamicin-containing media, a 5- to 6-log difference was observed between total cfu recovered at 30 C compared to 39 C (Table 11).

Both Tn551 and Tn4001 transpose at a frequency of about $10^{-4}$ (Luchansky and Pattee, 1984; Lyon et al., 1984). The transposition
frequencies (Table 11) of $1.63 \times 10^{-4}$ (ISP479) and $8.95 \times 10^{-4}$ (ISP1801) for Tn551 and Tn4001, respectively, are in agreement with published results. Strain ISP1504 (containing plasmid pPQ58) provided the unique opportunity to test simultaneously for transposition of Tn551 and Tn4001. Although Tn551 transposed at a "normal" frequency ($3.59 \times 10^{-4}$), transposition of Tn4001 from pPQ58 was not detected (Table 11). It is possible that one or both ends of Tn4001 were altered as a consequence of insertion onto pPQ58, or that the presence of Tn551 prohibited transposition of Tn4001 (e.g., transposon immunity). Since Tn4001 structurally resembles a Kleckner Class I transposable element (1.35-kb terminal repeats), and transposon immunity has been demonstrated only among Class II elements, it is more likely that damage to the ends of Tn4001 precluded transposition. From the results in Table 11, it was concluded that Tn917 and Tn4001 sequences cannot transpose from plasmid pPQ126.

To test the behavior of plasmid pPQ126 in different genetic backgrounds, pPQ126 was introduced into a recipient strain that lacked a chromosomal target (ISP1844), and recipient strains that contained a chromosomal target (ISP1846 and JBL85). A recombination-deficient strain (JBL177) was also used. Both phenotypic and molecular criteria were employed to determine if pPQ126 was maintained as an integrated replicon when the above strains were grown under non-permissive conditions. Cells possessing pPQ126 were resistant to 10 ug/ml each of erythromycin and gentamicin. In addition, cells containing autonomous pPQ126 were resistant to higher levels of chloramphenicol (between 11 and 14 ug/ml) than cells that contained a chromosomal copy of pPQ126 (about 10 ug/ml of
chloramphenicol), or cells that lost the plasmid (about 6 ug/ml of chloramphenicol). When the DNA from cells containing autonomous pPQ126 [Em^Gm^Cm^ (14 ug/ml)] was analyzed, both the 11.1-kb and 2.5-kb HindIII fragments of pPQ126 were discernible. When DNA extracted from cells that were resistant to 6 or 10 ug/ml of chloramphenicol was subjected to electrophoretic analysis, a typical chromosomal DNA digestion pattern was obtained, confirming the absence of autonomous pPQ126. Isolates that retained an integrated copy of plasmid pPQ126 were identified by their resistance to about 10 ug/ml of chloramphenicol, and by the absence of autonomous plasmid DNA.

Although there was no significant difference between the total number of cells recovered at 30 C compared to 39 C, plasmids pPQ125 and pTVIj-g were lost at elevated temperature on non-selective media (Tables 12 through 16). Colonies screened from the 39 C BHI plates were differentiated in two ways from their 30 C counterparts: 1) electrophoretic analysis demonstrated that autonomous pPQ126 was absent; and 2) the tolerance to chloramphenicol dropped from at least 11 ug/ml to only 6 ug/ml. In all strains tested, except JBL85, plasmid pPQ126 was lost from 5 to 8% of the cells grown on non-selective media at the permissive temperature (30 C). Even when erythromycin or gentamicin were included in the media, pPQ126 was eliminated from a population of cells grown at 39 C if homologous chromosomal sequences were not present (ISP1844; Table 12). Conversely, plasmid pPQ126 was retained by cells grown at 39 C on media containing either gentamicin or erythromycin, provided that Tn551 or Tn4001 target sequences were available as integration sites (Tables 13, 14, and 15).
pPQ126 integrated into a chromosomal Tn4001 target at a frequency of $5.76 \times 10^{-2}$ in a recombination-proficient host (JBL85), and at a frequency of $1.2 \times 10^{-5}$ in a recombination-deficient host (JBL177). These results suggest that integration of plasmid pPQ126 is highly dependent upon the recombination functions of the host cell. Retention of other integrable plasmids is also dependent on a functional rec$^+$ gene (Haldenwang et al., 1980; Niaudet et al., 1982). Considerably more Em$^-$ cfu were recovered at 39 C from JBL85 ($4.35 \times 10^7$) when compared to ISP1844 ($<1 \times 10^4$), because pPQ126 was retained in JBL85 due to integration into the Tn4001 target (compare Tables 13 and 15).

It was possible to test the effect of selection (gentamicin or erythromycin) on the integration of pPQ126 by using strain ISP1846 which contains the ermB317 mutation within Tn551. As shown in Table 15, the integration frequency was $2.2 \times 10^{-2}$ when selection was made for resistance to erythromycin, and $3.26 \times 10^{-5}$ when selection was made for resistance to gentamicin. The combination of gentamicin and elevated temperature retards the growth of cells containing an insertion of Tn4001 (J. B. Luchansky, Department of Microbiology, Iowa State University, Ames, Iowa, unpublished data; G. G. Mahairas, Department of Microbiology, Iowa State University, Ames, Iowa, personal communication), and this may explain the lowered frequency when selection was made for resistance to gentamicin. When possible, integration events were selected for on erythromycin-containing media to avoid this problem.

Since the frequency of integration (recombination) is also related to the extent of chromosomal homology (Duncan et al., 1978), it was not
surprising that pPQ126 did not integrate at an appreciably different rate into a chromosomal Tn551 target versus a Tn4001 target. Tn551, Tn917, and Tn4001 are about the same size, and comparable lengths of Tn917 and Tn4001 are available on pPQ126 to direct integration. Integration of pPQ126 occurred at similar frequency in JBL85 (5.76 x 10^{-2}; Tn4001) and ISP1846 (2.2 x 10^{-2}; Tn551) when selection was made for erythromycin resistance. In general, the integration frequency of pPQ126 was higher than the transposition frequency of Tn551, Tn917, or Tn4001 (compare Tables 11 and 17). An obvious advantage of a temperature-sensitive insertion vector is the high yield of cells containing an integrated plasmid. Once the vector is established (presumably in multiple copies), each cell should experience an integration event under non-permissive conditions. For typical insertion vectors (e.g., pJH101), recovery of an integrated plasmid is dependent upon the frequency of transformation, and competent-cell transformation of B. subtilis occurs at low efficiency (Dubnau, 1983; Gryczan, 1982).

Chromosomal DNA containing integrated pPQ126 (ISP1846) was transformed under non-permissive conditions into a plasmid-free recipient (ISP5) to determine if pPQ126 would transfer and remain integrated. Because competent cells of S. aureus are not readily transformed by plasmid DNA (Table 4), transformants possessing plasmid markers could only result from transformation with chromosomally integrated (not autonomous) pPQ126. Thirty-three transformants, comprising 8 distinct phenotypic classes, were recovered (Table 18). About 40% (13 of 33) demonstrated transfer of pPQ126 markers only, but the remainder harbored plasmid markers as well as other
donor markers (\textit{trp}, or \textit{tyr}, or \textit{thr}). The co-transfer of donor markers from the region of the Tn551 target suggests that \textit{pPQ126} was integrated at the specific Tn551 site in ISP1846. Furthermore, electrophoretic analysis demonstrated that \textit{pPQ126} remained integrated in these transformants. These results suggest that elevated temperature (39 C) selects and maintains \textit{pPQ126} in an integrated state.

To determine if integrated \textit{pPQ126} would transfer and remain integrated under permissive conditions, competent cells of ISP5 were transformed with high molecular weight DNA prepared from JBL85, and all incubations were carried out at 34 C. Three classes of transformants were obtained. All three classes contained some form of autonomous plasmid DNA (Table 19 and Figure 15). It was not clear, however, if \textit{pPQ126} integrated into the host chromosome prior to becoming autonomous, or if the plasmid circularized immediately upon entry. The recovery of these autonomous plasmids under permissive conditions further demonstrates the effects of temperature for the establishment and maintenance of integrated \textit{pPQ126}.

A strain containing an integrated copy of plasmid \textit{pPQ126} (JBL113) was grown at 30 C to determine if \textit{pPQ126} would revert to the autonomous state under permissive conditions. As a consequence of excision, a 7-kb HindIII fragment (in addition to the 11.1-kb and 2.5-kb fragments of \textit{pPQ126}) was observed following digestion of plasmid DNA extracted from cells of strain JBL113 grown at 30 C (Figure 14). Most recently, temperature-dependent integration/excision of plasmid \textit{pTRA117} (14.3 kb; Km\textsuperscript{R}) was reported in \textit{B. stearothermophilus} (Koizumi et al., 1986). In the presence of kanamycin, cells grown at 48 C harbored autonomous \textit{pTRA117}, whereas cells grown at 63
C contained pTRA117 integrated into the host chromosome. When cells containing integrated pTRA117 were grown at 48°C, a new and larger plasmid (designated pTRZ117) was observed. Plasmid pTRZ117 consists of pTRA117 plus an additional 1.8-kb segment from the host chromosome. Unlike the parental plasmid, pTRZ117 is stably maintained at 65°C. The pTRA117/pTRZ117 system (believed to be the first report of temperature-dependent integration into, and excision from, the host chromosome) appears similar to the situation described above with JBL113 (see below).

The restriction analyses of plasmid pPQ126 (Figure 16A) compared exactly with the published restriction map of the parental plasmid (pTV1es), after adjustment for the presence of the additional 2.5-kb HindIII fragment (the aaca-aphD gene of Tn4001). Ultimately, it was necessary to confirm that pPQ126 sequences were chromosomally integrated; however, the avidities of 32P-labeled pPQ132 and 32P-labeled pLUCH75 were initially tested against autonomous pPQ126. The GmR (pPQ132) and EmR (pLUCH75) probes were determined to be highly specific for only those sequences of pPQ126 that were precisely homologous to the aaca-aphD gene of Tn4001 and the ermB gene of Tn551 (Figure 16, panels B and C, respectively). The specificity of the EmR probe was most evident; 32P-labeled pLUCH75 did not hybridize with the 1.45-kb Xba fragment, even though this fragment contained the emr-distal terminal repeat of Tn917 (Figure 16C, lane 2). Although both ends of Tn917 (38 base-pairs inverted repeats) share some homology, the emr-proximal inverted repeat resembles the inverted repeats of Tn551 (which are identical) more closely than the
erm-distal inverted repeat of Tn917 (Perkins and Youngman, 1984). The data in Figure 16 demonstrated the high specificities of both probes (particularly pLUCH75) for homologous sequences of pPQ126.

The Gm$^*$ (panel B) and Em$^*$ (panel C) probes confirmed the presence of pPQ126 as an integrated replicon in JBL70, JBL71, and JBL74 (see Figure 17). Furthermore, the results in Figure 17 suggested that plasmid pPQ126 exists in either a different orientation or in a different position in the genome of JBL74 compared to JBL70 or JBL71. The differences in hybridization to JBL74 versus the other isolates may reflect: 1) a deletion occurred within pPQ126 or the adjacent chromosomal DNA as a consequence of integration and/or transformation, or 2) pPQ126 was integrated in the opposite orientation. It was also interesting to correlate the Gm$^S$ phenotype of JBL70 with the absence of hybridization to $^{32}$P-labeled pPQ132 (panel B, lanes 2 and 6). As mentioned previously, the Gm$^S$ phenotype most probably resulted from deletion of the $aacA-aphD$ gene of pPQ126 during integration and/or transformation.

The Gm$^*$ and Em$^*$ probes were also utilized to determine the origin of the additional HindIII fragments present in pLUCH88 and pLUCH89. From the results presented in Figure 18, it was concluded that all HindIII fragments of pLUCH89 were derived from plasmid pPQ126, or deleted derivatives of pPQ126 and/or pLUCH89 (lane 6, panels B and C). The hybridization data of Figure 18 did not, however, delineate the origin for 2 fragments of HindIII-digested pLUCH88 (lane 5, panels B and C). It is possible that the 6.5-kb and/or the 2.9-kb fragments of HindIII-digested pLUCH88 (or segments of these fragments) originated from the chromosome. To test this
hypothesis, \(^{32}\text{P}\)-labeled pLUCH88 was hybridized to ISP5 DNA. As shown in Figure 19, pLUCH88 did not hybridize with uncut or HindIII-digested ISP5 DNA (panel B, lanes 3 and 6, respectively). Because plasmid pLUCH88 DNAs were not of chromosomal origin, one must presume that this DNA was derived from pPQ126, or deleted derivatives of plasmids pPQ126 and/or pLUCH88.

Plasmid pPQ126 plus chromosomal DNA (pLUCH113) was excised from a chromosomal locus following growth of JBL113 at 30 C in the presence of erythromycin, gentamicin, and chloramphenicol (Figures 14 and 20). This process was designated plasmid disintegration. In addition to bands representing linear, OC, and multimeric configurations of pLUCH113, the uncut preparation of pLUCH113 contained 2 dense-staining (e.g., CCC) bands (Figure 20A, lane 4). It is possible that 2 plasmid species were generated following growth of JBL113 at 30 C: 1) the parental plasmid (pPQ126) resulting from precise disintegration; and/or 2) the parental plasmid (or a derivative thereof) plus an additional 7.0-kb segment of chromosomal DNA resulting from imprecise disintegration. Because the 7.0-kb fragment did not hybridize to pPQ126 sequences and because pLUCH113 DNA (but not pPQ126) hybridized to HindIII-digested fragment(s) of total DNA from ISP5 and ISP1565, it was concluded that the 7.0-kb fragment was derived from the chromosome (see Figure 20). The recovery of plasmid DNA plus chromosomal passenger fragments directly in \(S. aureus\) obviates cleavage of chromosomal DNA (containing integrated pPQ126) with restriction endonuclease(s) followed by religation and transformation into a Gram-positive host to clone \(S. aureus\) DNA using an integrable plasmid system.
Future studies should be directed to examine the location and mechanism of integration of pPQ126, and to characterize the additional plasmid DNA present following transformation and/or growth of cells containing integrated pPQ126 under permissive conditions. As described above, pPQ126 integrates into homologous chromosomal target sequences in response to elevated temperature, and pPQ126 is maintained as an integrated replicon at elevated temperature (Tables 13, 15, and 18, and Figure 17). It is also clear that pPQ126 excises from the chromosome under permissive conditions, and that excision can result in derivatives of pPQ126 (Table 19, and Figures 15, 18, and 19). Integration of pPQ126 into a defined locus followed by temperature-induced excision should prove to be a rapid and efficient means to clone specific segments of the S. aureus genome. The successful cloning of pPQ126 directly in S. aureus greatly facilitates the subsequent transfer (via transduction) of this vector into different genetic backgrounds. Compared to other mechanisms for transferring plasmid DNA, transduction is more efficient, less tedious, less expensive, and less time consuming to perform. The construction of a temperature-sensitive insertion vector that can be directed to multiple sites on the chromosome, should greatly facilitate genomic analysis of S. aureus.
SUMMARY

For several reasons, most notably a low-efficiency transformation system, digestion by nucleases, and the reliance on selection for resistance to chloramphenicol, it was not possible to adapt a well-characterized *B. subtilis* integrable plasmid (pJH101) for use in *S. aureus*. In an attempt to circumvent these problems, various parameters of the standard protoplast transformation protocol were modified to obtain higher transformation frequencies. The following conditions resulted in the greatest numbers of transformants: CsCl-purified plasmid DNA was presented to protoplasts that were heated at 56°C for 45 seconds immediately prior to the addition of DNA. Following a 3.5-h incubation at 30°C, the resulting mixture was plated onto R agar containing the selective agent incorporated directly into the medium. Frequencies as high as $3.08 \times 10^5$ transformants per ug of pC194 DNA were obtained with the improved protocol, compared to a frequency of $1.18 \times 10^6$ obtained with the unmodified procedure.

The improved protoplast transformation system and the use of a restriction-deficient recipient strain facilitated the isolation of the recombinant molecule pPQ126 directly in *S. aureus*. Plasmid pPQ126 is a novel, temperature-sensitive integrable plasmid composed of an 11.1-kb HindIII fragment from pTV1ts (encoding for resistance to erythromycin and chloramphenicol, and containing a temperature-sensitive origin of replication) and a 2.5-kb HindIII fragment from transposon Tn4001 (encoding for resistance to gentamicin). When a population of cells containing pPQ126 is grown under non-permissive conditions (39°C) and selection is
made for retention of the plasmid (selection for resistance to gentamicin, erythromycin, and chloramphenicol), isolates can be obtained in which pPQ126 has integrated into homologous chromosomal target sequences. Portions of Tn917 and Tn4001 on pPQ126 direct the homology-dependent integration into specific chromosomal copies of Tn551 or Tn4001. Plasmid pPQ126 integrates at frequencies of $2.2 \times 10^{-2}$ and $5.76 \times 10^{-2}$ into chromosomal Tn551 and Tn4001 targets, respectively. Integration of pPQ126 does not occur in cells lacking homologous target sequences. Integration of pPQ126 occurs at significantly reduced frequency in a recombination-deficient host. Elevated temperature is required to establish and maintain pPQ126 as an integrated replicon. Under permissive conditions, plasmid pPQ126 can excise from the chromosome, and excision can produce derivatives of pPQ126 that harbor DNA fragments of chromosomal origin. As a new approach for genetic transfer and analysis, pPQ126 should prove useful for both mapping and cloning various genes in S. aureus.
LITERATURE CITED


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PRESS ON

Nothing in the world can take the place of persistence. Talent alone will not; nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education alone will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent.

(Calvin Coolidge)

Let us not look back in anger, nor forward in fear, but around in awareness.

(James Thurber)