Plasmids, Drug Resistance, and Gene Transfer in the Genus *Streptococcus*

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INTRODUCTION

The streptococci represent a large group of fastidious gram-positive microorganisms of remarkable heterogeneity. Most streptococci are facultative anaerobes and nonmotile, and they tend to grow in chains of variable length, especially during cultivation in vitro.

The role of streptococci in suppurative diseases, such as pharyngitis (strept throat), scarlet fever, erysipelas, cellulitis of the skin, impetigo, and lymphangitis, is well known. Related non-suppurative diseases include acute glomerulonephritis and acute rheumatic fever. Most of these illnesses are associated with infections by *Streptococcus pyogenes* (Lancefield group A), although other streptococcal species are sometimes involved. The viridans group (e.g., *Streptococcus sanguis*, *Streptococcus mutans*, *Streptococcus salivarius*, and *Streptococcus mitior*) inhabit the oral cavity and commonly are involved in cases of subacute bacterial endocarditis. Dental caries, one of the most widespread bacterial infections in humans, is believed to involve *S. mutans* (91). *Streptococcus faecalis* (group D), a normal inhabitant of the gut, can be involved in endocarditis and is commonly involved in urinary tract infections. Group D strains are also frequently implicated in root canal infections (76, 235), and some strains are cariogenic in germfree rats (18, 68, 181, 227). In recent years, *Streptococcus agalactiae* (group B) has become one of the common causes of meningitis in newborn infants (6, 244). As the etiological agent of lobar pneumonia, *Streptococcus pneumoniae* is a major killer of humans, albeit its lethality frequently arises as a complication of a preceding debilitating illness. In addition to their medical importance, the streptococci play highly significant roles in the dairy industry; *Streptococcus lactis*, *Streptococcus cremoris*, and other streptococci are intimately involved in the production of cheeses and creams.

The first reports of plasmid deoxyribonucleic acid (DNA) in streptococci came from two independent studies, both of which were described about 9 years ago. First, Courvalin and co-workers (52) reported satellite DNA in lysates of a drug-resistant strain of *S. faecalis* centrifuged to equilibrium in CsCl gradients containing ethidium bromide. No satellite (plasmid) DNA was observed in a drug-sensitive variant of this strain. Second, a small plasmid in *S. mutans* was identified by Dunny et al. (68); in this case, the plasmid was physically characterized. Since then, plasmids have been identified in numerous strains of streptococci; a list of the plasmids that have been at least partially characterized and named is given in Table 1.

In general, plasmids are as common in streptococci as they are in gram-negative enteric bacteria; however, they are found more easily in some species than in others. In the case of *S. faecalis* (B. Brown, Y. Yagi, and D. B. Clewell, unpublished data) and *S. lactis* (116, 129), it is not uncommon to find strains containing five or more plasmids, and plasmid-free isolates are
### Table 1. List of streptococcal plasmids

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* Abbreviations: Tc, tetracycline; Em, erythromycin; Sm, streptomycin; Km, kanamycin; Nm, neomycin; Gm, gentamicin; Cm, chloramphenicol; Asa, arsenate; Asi, arsenite; Cr, chromate; UV, ultraviolet light; Hly-Bac, hemolysin-bacteriocin; Bac, bacteriocin; PR, pheromone response; Lac, lactose utilization; Prt, protease production; Suc, sucrose utilization; Cit, citrate utilization.

rare. In contrast, plasmids seem to be less frequent in isolates of *S. mutans* (68, 143, 145, 193) and *S. pneumoniae* (25, 57, 194, 205, 210, 243).

As Table 1 shows, plasmids determine a variety of different functions, including conjugal activity, drug resistance, hemolysins, proteases, bacteriocins, bacteriocin sensitivity, resistance to ultraviolet light, resistance to arsenate, arsenite, and chromate, and utilization of lactose, sucrose, and citrate. Although specific plasmids have not been characterized yet, the involvement of plasmids has also been implicated in streptococcin A-FF22 production (214), nisin production (84, 131), galactose and xylose fermentations (131), and production of M proteins and serum opacity factor (30).

Plasmid sizes range from a few megadaltons (Mdal) to as much as 76 Mdal; more than 85% of the plasmids that have been described are smaller than 40 Mdal. Copy number is usually high (10 to 30 copies per chromosomal genome equivalent) in the case of plasmids smaller than about 7 Mdal and generally low (1 to 2 copies per chromosomal gene equivalent) in the case of larger plasmids.

In a few cases, workers have found that plasmids are responsible for properties which relate to species nomenclature. For example, *Streptococcus faecalis* subsp. *zymogenes* (sometimes called *Streptococcus zymogenes*) is so named because of its production of hemolysin; in fact, most of these organisms are simply *S. faecalis* strains bearing plasmids which determine hemolysin expression. Similarly, plasmids determining citrate utilization appear to be responsible for the name *Streptococcus lactis* subsp. *diacetylactis* (or *Streptococcus diacetylactis*) (117).

Thus far, good evidence for plasmid involvement in pathogenicity has not been found; however, in many cases knowledge of what bacterial properties do in fact contribute to pathogenicity and thus the ability to know what to look for are at best very limited. (A drug-resistant strain...
of *S. pyogenes* [strain AC-1] cured of its only detectable plasmid [an R-plasmid] maintained its M22 and T12 surface antigens intact (36). M antigen is generally involved in pathogenicity. Despite suggestions (30) that M protein and serum opacity expression might be plasmid determined, physical evidence for plasmid DNA is still lacking.

In the case of the hemolytic strains of *S. faecalis*, it has never been shown that plasmid-determined hemolysin contributes to pathogenicity in human infections. And unlike the situation with *Escherichia coli* (48, 67, 167), there have been no reports that urinary tract infections caused by this species are more likely to involve hemolytic strains. It is interesting that the hemolysin protein also has bacteriocin activity (9, 23, 89).

Higuchi et al. (99, 100) reported that *S. mutans* strains PK1 and JC-2 harbor plasmids that relate to glucan production, which is believed to contribute to cariogenicity. However, efforts to confirm these observations in other laboratories have been unsuccessful (36, 68, 143, 193). Indeed, only about 5% of the *S. mutans* strains examined have been found to harbor plasmids (143). Macrina and Scott (144) showed that small cryptic plasmids found in four different human clinical isolates were all very similar, if not identical, with regard to size (3.6 Mdal) and sensitivity to several restriction endonucleases. Glucosyltransferase-deficient mutants (defective in glucan production) of one of these strains (strain LM7) still harbored such a plasmid (64).

In the case of the lactic streptococci (group N), which are so important in dairy fermentations, the ability to coagulate milk successfully is very dependent on the ability of the organism to metabolize lactose and to break down casein. There is good evidence (Table 1) that these two properties are related to the presence of specific plasmids.

**PURIFICATION OF PLASMID DEOXYRIBONUCLEIC ACID FROM STREPTOCOCCI**

A number of procedures have been used to isolate plasmids from different types of streptococci; most of these procedures are adaptations of methods developed for gram-negative bacteria. In general, the streptococci are somewhat difficult to lyse and usually require extended incubation in lysozyme or other lytic enzymes beforeionic detergents, such as sodium dodecyl sulfate and Sarkosyl, bring about lysis. Even under these conditions, lysis is frequently incomplete. *S. faecalis* is usually an exception, readily yielding clear and highly viscous lysates (41). *S. faecalis* has also been lysed effectively with a Brij-deoxycholate detergent mix after lysozyme exposure (52; M. Mikus and D. B. Clewell, unpublished data).

In the case of other species, such as those belonging to the viridans group, growth of the cells in the presence of 20 mM DL-threonine (29, 133) or 2 to 5% glycine (193) ultimately leads to better lysis. This is presumed to be due to a weakening of the cell wall structure during growth in these agents. Growth in DL-threonine has also been used with group N streptococci (118, 136).

Pronase has been useful (41, 42a) in the preparation of lysates and probably aids in breaking up DNA-protein-membrane complexes after lysis. Along with controlled shearing of the lysate (44), this facilitates the removal of stringy debris, which can interfere with the collection of buoyant density gradients run subsequently.

In group A streptococci, a specific phage-associated lysisin has been useful in the generation of lysates (154). Mutanolysin, an enzyme found in culture filtrates of *Streptomyces globisporus*, is very effective in lysing *S. mutans* in the absence of detergents (242), and there is evidence that this enzyme greatly facilitates the detection of plasmid DNA in this species (115). Mutanolysin also effectively lyzes other members of the viridans group (242), and Tomich (unpublished data) has shown that this enzyme effectively lyzes *S. faecalis*. Recently, Siegel et al. (206) have reported the use of mutanolysin in the preparation of *S. mutans* protoplasts.

Lysates can be centrifuged directly in CsCl-ethidium bromide buoyant density gradients, and this results in the separation of covalently closed circular plasmid DNA molecules from the linear chromosomal fragments. The covalently closed circular DNA appears as a satellite band which has a higher density than the chromosomal DNA band. This material can then be analyzed by agarose gel electrophoresis, sucrose density gradient centrifugation, or electron microscopy.

Whereas the efficient recovery of DNA from *S. faecalis* allows the direct use of crude lysates in CsCl-ethidium bromide gradients (41), other species frequently require a plasmid enrichment step before buoyant density centrifugation. This can be accomplished by performing what is commonly referred to as a clearing spin, a low-speed (20,000 × g to 30,000 × g) centrifugation which selectively pellets chromosomal-membrane complexes. This method has been used frequently with Brij-deoxycholate or Triton X-100 lysates of plasmid-bearing gram-negative bacteria (43, 44), and it can also be used with Sarkosyl or...
sodium dodecyl sulfate (53, 68) lysates of streptococci. In the case of Sarkosyl lysates, a well-defined pellet is usually not obtained by centrifugation; rather, a loose gelatinous material appears as the sediment. However, carefully removed supernatant is enriched in plasmid DNA. When sodium dodecyl sulfate is used, the lysates may be brought to 1 M NaCl and allowed to stand in the cold (0°C) for a few hours or overnight before centrifugation (50, 68). The sodium dodecyl sulfate, which precipitates in the cold, aids in the selective sedimentation of chromosomal DNA.

Cleared lysates can be centrifuged directly in CsCl-ethidium bromide gradients; this method has the advantage that a relatively large volume of material can be used in a single centrifuge tube. However, when clearing spins are performed, if the crude lysate is too concentrated or too viscous, entrapment of plasmid DNA by sedimenting chromosomal material may cancel out the advantages of desired enrichment (i.e., enrichment may occur, but at a significant sacrifice of plasmid DNA).

In some cases, investigators have phenolized crude or cleared lysates and concentrated DNA with ethanol before CsCl-ethidium bromide banding (35). This allows the addition of relatively large amounts of DNA to the gradients without the debris problem that is encountered frequently when too much crude lysate is used. In the case of phenolized cleared lysates, material may be analyzed directly by agarose gel electrophoresis.

A plasmid purification protocol described by Currier and Nester (55) for Agrobacterium is useful in the preparation of plasmid DNA from streptococci (133). This method involves an alkaline denaturation step which, after neutralization, leaves covalently closed circular DNA as a duplex molecule. Chromosomal DNA fragments, which are not able to renature because of their linearity, can be removed selectively by phenolization in the presence of 1 M NaCl. The resulting aqueous phase frequently contains more plasmid DNA than chromosomal DNA.

A recently developed procedure reported by LeBlanc and Lee (136) combines certain steps from other procedures (55, 90, 95) and has been used with several streptococcal species. After lysates are subjected to an alkaline denaturation-reneutralization step, 2% sodium dodecyl sulfate and 1 M NaCl are added; then after the preparation stands overnight in the cold, high-molecular-weight (chromosomal) DNA is pelleted with the precipitated sodium dodecyl sulfate. The supernatant is treated with ribonuclease and extracted with phenol. After concentration with ethanol, the sample, which is highly enriched for plasmid DNA, is ready for analysis by agarose gel electrophoresis.

Macrina et al. (147) have described a simple analytical procedure for rapid screening for small plasmids in oral streptococci. Insofar as this method does not remove chromosomal DNA, plasmids larger than 9 to 10 Mdal are not easily detectable; however, this procedure should be ideal for screening chimeric plasmids (see below), which are usually in this small size range.

CONJUGATION AND PLASMID TRANSFER

In 1964 (192) there was a report which indicated a high frequency of conjugal transfer (2.2 per donor) of a chloramphenicol resistance mutation (presumably on the chromosome) in S. faecalis; however, since that time there have not been reports of additional examples of chromosomal transfer at such a high frequency. It was not until 9 years later that conjugal transfer was reported again, again in S. faecalis. Tomura et al. (221) observed the transfer of a hemolysin-bacteriocin determinant at a relatively high frequency (up to 5.8 × 10⁻² per donor), and whereas direct evidence for a plasmid bearing this property was not provided, it is likely that this was indeed the case. (As Table 1 shows, there are now numerous examples of hemolysin-determining conjugative plasmids in S. faecalis.) At about the same time, Jacob and Hobbs (112) presented evidence for conjugal transfer of multiple drug resistance from S. faecalis strain JH1, and these workers were the first to show the direct involvement of plasmid DNA. Two conjugative plasmids, pJH1 (multiple resistance) and pJH2 (hemolysin-bacteriocin) (111), were identified in this strain. In both of the above-described reports (112, 221), transfer occurred in broth in a matter of a few hours. Transfer was deoxyribonuclease resistant, and the authors provided evidence against transduction. Thus, cell-to-cell contact seemed to be a requirement for transfer.

Additional evidence for the presence of conjugative plasmids in S. faecalis, as well as in other streptococci, soon followed (Table 1); furthermore, it was shown that some of these plasmids could mobilize nonconjugative plasmids (24, 70, 179, 211, 220) and even chromosomal markers (80, 81). The conjugative hemolysin plasmid pAD1 of S. faecalis has been mapped physically (Fig. 1).

Some attention has been focused on pAMβ1, a 17-Mdal conjugative plasmid that determines erythromycin resistance. This resistance is representative of the so-called MLS phenotype
(230) (i.e., resistance to macrolides, lincosamides, and streptogramin B). Originally identified in S. faecalis strain DS5 (41), pAMβ1 has been shown to have a broad host range. The transferability of this plasmid was first shown by Hershfield, and its transfer into nine different species of streptococci has now been demonstrated (85, 87, 98, 134, 153). In addition, it transfers into Lactobacillus casei (87), Staphylococcus aureus (75, 200a), and Bacillus subtilis (128). Interestingly, in S. faecalis the transferability of pAMβ1 is inhibited dramatically if pAMγ1 or pAD1 is also present in the donor strain, even though the latter two plasmids are highly transmissible (B. Brown and D. B. Clewell, unpublished data). This explains the original failure to observe pAMβ1 transfer from strain DS5 (70) (i.e., the presence of pAMγ1 inhibited transfer).

MLS resistance plasmids resembling pAMβ1 in size (15 to 20 Mdal) have been identified in S. faecalis (49, 53, 226), S. pyogenes (12, 19, 35, 154), and S. agalactiae (74, 98, 104, 107), as well as in Lancefield groups C and G (20). Malke published (153) a rather comprehensive study which showed that several MLS resistance plasmids (pAMβ1, pDC10535, pIP501, and pSM15346) from different sources transfer on filter membranes among strains belonging to groups A, B, D, and H, and Hershfield (98) and Horodniceanu et al. (107) identified plasmids in group B strains which could be transferred to group B, D, F, and H strains. R-plasmid transfers between S. pneumoniae and streptococcal groups A, B, and D (75, 211) and between S. aureus and groups A, B, and D (75, 200a) have also been reported.

Conjugative systems that recently have been described in group N streptococci (86, 163) involve the transfer of plasmids which determine the ability to metabolize lactose. Interestingly, some of the resulting transconjugants exhibit an unusual cell aggregation phenotype and donate Lac at a high frequency; in one case, such strains harbor an enlarged Lac plasmid (227a).
SEX PHEROMONES IN STREPTOCOCCUS FAECALIS

In S. faecalis, conjugative plasmids can be placed into two general categories. There are plasmids such as pAD1, pOB1, pPD1, pJH2, pAMy1, pAMy2, and pAMy3, which transfer at a relatively high frequency (10⁻³ to 10⁻¹ per donor) in broth (33, 69, 71, 111; Y. Yagi, B. Brown, and D. B. Clewell, unpublished data), and there are plasmids such as pAMβ1, pAC1, pIP501, and pSM15346, which transfer poorly in broth (usually less than 10⁻⁶ per donor) but rather efficiently (10⁻⁴ to 10⁻² per donor) when the matings are carried out on filter membranes (98, 153; Brown and Clewell, unpublished data). The reason for these differences is now becoming clear. Those systems which transfer readily in broth make use of sex pheromones to generate cell-to-cell contact, whereas those that transfer poorly do not (Brown and Clewell, unpublished data). As Fig. 2 shows, recipient strains excrete soluble, protease-sensitive, heat-stable substances which induce certain donor cells to become adherent (33, 34, 69, 71). This induction facilitates the formation of donor-recipient mating aggregates that arise from random collisions. (S. faecalis is usually nonmotile.) Since a cell-free filtrate of a recipient also elicits an aggregation (clumping) response when it is mixed with donors, this substance has been referred to as clumping-inducing agent (CIA). Usually 30 to 45 min is required before visible clumping is observed, and short (10-min) incubations of donors with recipients results in very little plasmid transfer. However, when a recipient filtrate is mixed with donor cells for 20 to 50 min before a short (10-min) mating, the frequency of plasmid transfer is increased by several orders of magnitude. Therefore, CIA can be viewed as a sex pheromone. The response of donor cells to CIA requires both ribonucleic acid and protein synthesis, but not DNA synthesis (69). The acquisition of a conjugative plasmid results in a shutting off of endogenous CIA production, and a cell with a newly acquired plasmid becomes responsive to exogenous CIA.

Interestingly, donors harboring different conjugative plasmids respond to different CIAS (71). A given recipient actually produces multiple pheromones. The acquisition of a given plasmid shuts off production of only the related pheromone, whereas the cell continues to produce other pheromones which can induce other donors with different conjugative plasmids. The pheromones are now indentified by relating them to the plasmids originally used to detect them (71). Thus, cPD1 refers to the CIA to which strains harboring pPD1 respond. Similarly, other activities are identified as cAMy1, cOB1, etc.

Studies have shown that, in addition to an aggregation response, a pheromone induces a function(s) that is related more directly to plasmid transfer (32). This was revealed by analyzing isogenic donor-donor matings, using derivatives of pAD1 containing two distinguishable transposons (Tn916 [79, 80] and Tn917 [218, 219]). It was reasoned that if the sole function of the pheromone (cAD1) was to induce aggregation, then once the cells made contact, transfer should have occurred equally well in both directions, regardless of which donor was induced with cAD1 before mating. However, it was found that when only one of the donors was induced, transfer occurred only in the direction from the induced strain to the uninduced strain. If both donors were induced, transfer occurred in both directions. Thus, the pheromone must also induce a preparation for plasmid transfer, the nature of which is not known. Conceivably, the pheromone induces a polycistronic operon (perhaps somewhat analogous to the Tra operon of certain conjugative plasmids in gram-negative bacteria [1, 234]), which, in addition to having determinants related to aggregation, also determines functions related to transfer.

Pheromone activity can be quantitated by using a simple microtiter plate system (71); serial twofold dilutions are used, and the highest dilution of filtrate that still induces clumping of the appropriate responder (donor) cells is taken to represent the pheromone titer. The titer for a given filtrate varies somewhat with the particular responder system; this depends on the conjugative plasmid, as well as the host. Typically, titers range from 4 to 64. One might argue that

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**Fig. 2. Expression of sex pheromone by a recipient S. faecalis strain and response by a donor containing a pheromone-sensitive conjugative plasmid.**
it would be disadvantageous to produce too much sex pheromone or that it would be disadvantageous for donors to be too sensitive; such behavior would result in donors becoming induced when they are too far away from recipients to make contact by random collision.

Dunny et al. found that the production of pheromone by recipient cells closely paralleled cell growth (71). In the case of certain plasmid-free strains, such as JH2-2 and DR1, the CIA activity in filtrates leveled off as cells entered the stationary phase. In the case of other strains, such as OG1 and ND539 (both Streptococcus liquefaciens subspecies), the activity in filtrates rapidly disappeared as the cells entered the stationary phase. It is likely that this decrease was due to degradation by the protease (gelatinase) produced by these strains (characteristic of the S. liquefaciens subspecies). A mutant of strain OG1 which does not degrade gelatin produces CIA activity, which does not decrease as the cells enter the stationary phase (Y. Yagi, R. Craig, and D. B. Clewell, unpublished data).

Recent data have shown that pheromone-induced donor cells have a new antigen on their surfaces (240a). A highly specific rabbit antiserum prepared against induced pPD1-containing cells (the serum was absorbed with uninduced cells) readily cross-reacted with different donors (induced) which harbored several different conjugative plasmids (pAD1, pOB1, pAMy1, pAMy2, and pAMy3). The surface material has been referred to as aggregation substance, and since it is sensitive to trypsin and pronase, this aggregation substance appears to be proteinaceous. When preparations were subjected to specific immunological staining procedures involving conjugated horseradish peroxidase and then analyzed by electron microscopy, an amorphous surface material (presumably aggregation substance) was observed (Fig. 3) on the surfaces of induced cells but not on uninduced cells (Y. Yagi and D. B. Clewell, manuscript in preparation). Pilus-like structures were not observed; however, the possibility that small microfimbriae that are difficult to resolve coat the surface remains. In the absence of immunological strains, no surface differences between induced and uninduced cells could be detected by either transmission or scanning electron microscopy (G. Dunny, Ph.D. thesis, University of Michigan, Ann Arbor, 1978; D. Birdsall, unpublished data).

It is presumed that the aggregation substance binds to a specific substance, designated binding substance, which is located on the surfaces of both recipients and donors (71). The interaction between aggregation substance and binding substance which results in aggregation requires divalent cations (e.g., Mg²⁺, Mn²⁺, Ca²⁺, or Co²⁺) and, interestingly, also phosphate ions (Yagi and Clewell, manuscript in preparation). However, there is no requirement for cations or phosphate if the induced cells are exposed to a pH between 2 and 3.

Kroghstad et al. (123) recently presented electron micrographs of mating mixtures of S. faecalis, showing what appear to be intercellular connections between chains of streptococci in the absence of fimbiae or pili. (Although the latter system represented a high-frequency transfer system, evidence for pheromone involvement was lacking.) Similar connections have been observed in pheromone-induced aggregates of cells harboring pPDI (Dunny, Ph.D. thesis); however, preparations of uninduced cells also have shown such connections. Thus, in this case at least, it was not clear whether the observed connections were an actual reflection of conjugal contact or an artifact of the preparation.

(It is noteworthy that in fact there have been a few reports dealing with fimbiae in streptococci [45, 92, 93, 97], and although it is not pointed out in their report, Tomura et al. [221] displayed an electron micrograph of a strain of S. faecalis which appears to possess polar fimbiae.)

The chemical nature of the sex pheromones is currently being examined. The sensitivity of the pheromones to proteases (including exopeptidases [R. Craig, unpublished data]) and their heat stability and dialyzability suggest that these compounds are small peptides. (It was reported originally that CIA was sensitive to trypsin [69].) However, subsequent studies have shown that this was probably due to chymotrypsin contamination. Purer preparations of trypsin failed to inactivate cPDI, cAD1, cAMy1, or cOB1, whereas chymotrypsin inactivated all of these activities (R. Craig and D. B. Clewell, unpublished data). Analyses of cPDI on columns have suggested a molecular weight of less than 1,000 (34).

An examination of 100 clinical isolates of S. faecalis showed that 34% exhibited a CIA response to a filtrate of the plasmid-free strain OG1-10 and that 72% excreted cPDI (71). Interestingly, the ability to respond to and to produce CIA activities was significantly more frequent among strains resistant to one or more drugs than among drug-sensitive strains (71). Thus, pheromones may contribute to the evolution of drug resistance in S. faecalis. A recipient producing numerous sex pheromones would probably be a prime target for R-plasmids which confer pheromone responses or which can be
mobilized by such systems. (It is noteworthy that pAMγ1, pAMγ2, and pAMγ3 [all originating in S. faecalis DS5 and having nearly identical molecular weights] each determines a response to a different pheromone [Y. Yagi, B. Brown, R. Craig and D. B. Clewell, unpublished data]. Thus, strain DS5 appears to be capable of responding to three different pheromones simultaneously.)

Whereas several of the above-mentioned pheromone-responding plasmid systems (pAD1, pAMγ1, pOB1, and pJH2) determine hemolysin, this phenotype is not necessarily related to the ability to respond. For example, pPD1, pAMγ2, and pAMγ3 do not determine hemolysin, but they confer a pheromone response (Brown, Yagi, and Clewell, unpublished data). (Although it was believed previously that pPD1 determined a hemolysin [71], recently this has been shown not to be the case. It is now known that in the original isolate [strain 39-5], hemolysin is actually determined by a different conjugative plasmid, pPD5, which has a similar molecular weight and frequently transfers together with pPD1 [Brown, Yagi, and Clewell, unpublished data]. However, pPD1 does determine a bacteriocin activity.) Also, of the 34 above-mentioned clinical isolates which exhibited CIA responses, only 9 were hemolytic.

A model (71) has been proposed (Fig. 4) to explain the relationship among plasmids, pheromones, and the aggregation phenomenon. This model schematically shows a plasmid-free recipient strain that produces two different pheromones, cA and cB; two isogenic donor strains harboring the conjugative plasmids pA and pB.
are also present. All three strains have the chromosomally determined binding substance. Plasmid pA determines the ability to respond to cA and, at the same time, through an IcA (inhibitor of cA) gene prevents production of endogenous cA. (Alternatively, inactivation of cA could be involved.) Similarly, plasmid pB allows its host to respond to cB and prevents the production of endogenous cB via gene IcB. The response of the donor cell to the pheromone is shown as an interaction (direct or indirect) of the latter with a responding substance (repressor or activator?) determined by gene RcA or RcB which, in turn, activates aggregation substance synthesis. Aggregation substance, which could be either plasmid determined (as depicted in Fig. 4) or chromosomally determined, locates itself on the cell surface, where it can recognize binding substance. (It is clear from the model how induced donors can self-aggregate, as well as bind to recipients.)

It should be possible to test this model genetically by obtaining mutations in the proposed determinants, and such efforts are currently under way. The conjugative hemolysin plasmid pAD1 is being used for this, since much is already known about its structure (Fig. 1), and the transposons Tn916 and Tn917 (see below) are proving to be useful as mutagenic insertion elements (Y. Ike, personal communication).

The fact that a single recipient strain of S. faecalis may produce numerous sex pheromones specific for different donors seems surprising at first, since it is possible that such cells have never before encountered the related plasmids. Conceivably, the pheromones may have other functions in the recipient cells or may represent degradation products of larger proteins. Plasmids may have evolved in such a way as to take advantage of such molecules as mating signals to facilitate their dissemination.

**RESISTANCE TRANSFER IN THE ABSENCE OF PLASMID DEOXYRIBONUCLEIC ACID: EVIDENCE FOR CONJUGATIVE TRANPOSONS?**

When multiply resistant clinical isolates of *S. pneumoniae* began to appear a few years ago (27, 113), efforts by several research groups to reveal R-plasmids were unsuccessful (25, 57, 194, 205, 210, 243; Brown and Clewell, unpublished data). Recently, there have been reports which show that resistance determinants in *S. pneumoniae* are capable of transfer to recipient strains on membrane filters by a deoxyribonuclease-resistant process (25, 204). Similar observations have been made in *S. faecalis* (79, 80), in group A, B, F, and G streptococci (105), and in certain oral streptococci (129a).

Shoemaker et al. (204) reported that two plasmid-free isolates of *S. pneumoniae* (strains BM6001 and N77) could transfer tetracycline and chloramphenicol resistance determinants on membrane filters at a frequency of $10^{-6}$ per donor. (Mating using an agar overlay improved...
the frequency 10- to 100-fold (210a). This transfer was resistant to deoxyribonuclease; however, transfer of an Em' chromosomal mutation (ery2) marker could be eliminated by deoxyribonuclease. The two resistance markers cat and tet had been shown previously by transformation studies to be closely linked, and it was also shown both physically and genetically that these markers represent insertions (referred to as Ω cat tet) in the bacterial chromosome (205). In matings, about 90% cotransfer of cat and tet was generally observed; occasionally tet transferred without cat, but the reverse did not occur (204). Also, Cm' Te' derivatives could be generated by transformation, but these derivatives failed to donate cat by conjugation. It was estimated that cat was 4 to 6 kilobases long, whereas tet was more than 30 kilobases long (205).

Buño-hoi and Horodniceanu (25) reported that several plasmid-free clinical isolates of S. pneumoniae could transfer resistance traits not only into S. pneumoniae recipients by filter mating, but also into group B and D strains. In some of these cases, transfer occurred in bloc as: (i) Te' and Cm'; (ii) Te' and MLS'; or (iii) Te', Cm', MLS', and Km'. Similar observations were made for clinical isolates of group A, B, F, and G streptococci (105).

Franke and Clewell (79, 80) have reported that a transferable tetracycline resistance determinant located on the chromosome of S. faecalis strain DS16 is located on a 10-Mdal transposon. Designated Tn916, this element inserted at multiple sites into several different conjugative plasmids. Transposition of Tn916 from the chromosome to the conjugative plasmid pAD1 is Rec independent, as is its ability to transfer in the absence of plasmid DNA (at a frequency of ~10-8). (Transfer of tetracycline resistance was not reduced if either the donor or the recipient was Rec-.) Transfer involved the entire transposon; after introduction of a conjugative plasmid into transconjugants, typical transposition to plasmid DNA could be detected. Transfer from a plasmid-free donor required cell-to-cell contact; extensive efforts to implicate transformation or transduction by a variety of means were unsuccessful. It is noteworthy that S. faecalis has never been reported to transform; a recent screening of 200 clinical isolates for the ability to transform (using pAMβ1, pAMα1, and a chromosomal Sm' mutational marker) under conditions where S. sanguis strain Challis readily transformed was totally negative (L. Dempsey and D. B. Clewell, unpublished data). In addition, transducing phages have never been reported in S. faecalis.

After transfer from the plasmid-free strain DS16C3, some transconjugants retransfer Tn916 at an elevated frequency (~10-6), about 100-fold higher than normal (86a). Interestingly, the transposition frequency from the chromosome to a subsequently introduced pAD1 is also elevated about 100-fold in such strains, suggesting a common step for both transfer and transposition.

It has also been shown that after Tn916 transfer, insertion can occur at different sites on the recipient chromosome (86a). This was done in the following way. Insofar as Tn916 has a single HindIII cleavage site, HindIII digests of chromosomal DNA containing Tn916 should give rise to two fragments, X and Y, which constitute the transposon-host DNA junction fragments. When the Southern blot hybridization technique is used and an EcoRI fragment of pAD1::Tn916 (there are no EcoRI sites in Tn916) is used as a probe, hybridization with the two fragments (X and Y) occurs readily. However, the size of the detectable X and Y fragments varies greatly in chromosomal DNA preparations obtained from different transconjugants (including those from secondary matings), a result which would be expected if Tn916 were located (inserted) at different sites on the chromosome. It is noteworthy that this result also provides strong evidence against the location of Tn916 on a plasmid which has escaped physical detection. If Tn916 were on such a plasmid, the X and Y fragment profile would be expected to remain the same in all transconjugants and would resemble the profile of donor cells. This is clearly not the case.

It is likely that Tn916 determines functions related to its own transfer, at least in part; it is large enough (10 Mdal) so that there would be room for such genetic information. Thus, transfer could simply represent an elaborate transposition event where the donor and recipient replicons are in different cells. A model based on an earlier proposal (79, 80) is shown in Fig. 5, where the transposon is excised and then can (i) reinsert into the chromosome (perhaps at a different location), (ii) insert into a resident plasmid, or (iii) transfer to another cell. After transfer into the recipient, insertion might be facili-
tated by zygotic induction of an integrase (the related transposase?). Since transfer might occur by a single-strand (i.e., plasmid-like) process, a copy of the transposon could remain in the donor and might still be capable of reinserting into host DNA. Proof for this hypothesis (Fig. 5) awaits further study.

In view of the evidence in a number of streptococcal species for conjugal transfer in a plasmid-free environment, it will be interesting to determine the extent to which these systems represent "conjugative transposons." In this regard, Guild et al. have shown recently (M. Smith, M. Hazum, and W. Guild, submitted for publication) that Tn916 has homology with the transferrable tetracycline element that they have studied in S. pneumoniae (204). In turn, the latter element had homology with similar determinants from nine other clinical isolates of pneumococcus and from group B strains. Studies on the homology of tetracycline resistance elements per se are currently being conducted by V. Burdett (submitted for publication).

TRANSDUCTION

Bacteriophage-mediated transduction occurs in group A, C, G (47, 110, 137, 148, 149, 151, 212, 213, 224, 228) and N (2, 161, 164, 198) streptococci, as well as in pneumococci (188), and the erythrogenic toxin associated with group A strains causing scarlet fever has long been known to be associated with a phage genome (245, 246). Recently, Totolian (222) reported the transduction of S. pyogenes determinants of M protein and serum opacity factor. Transducing phages have never been reported in group B or D streptococci, but it is not clear whether this simply reflects the absence of a search for such systems.

Higuchi et al. (101, 102) reported that certain mitomycin C-exposed strains of S. mutans undergo lysis and give rise to defective phage particles. These particles, as well as their released DNA, reportedly transformed S. sanguis and variant S. mutans strains into producers of insoluble polysaccharide (glucan); however, peculiarities regarding certain phenotypic properties (e.g., the ability to produce ammonia from arginine by the transformant but not by the recipient or by the original phage-containing strain) raises the possibility of alternative interpretations of the data.

Transduction of the ERL1 (Em') plasmid among group A streptococci (150, 151) and transduction of this plasmid from group A to groups C and G (156, 207) and back to group A (207) suggest that this means of gene transfer may be important in the spread of plasmid-mediated resistance in these organisms. In S. lactis, transduction of a plasmid determining the ability to utilize lactose has been reported (162, 172).

TRANSFORMATION

Transformation occurs in streptococcal groups F, H, N, and O (63, 186, 232), S. pneumoniae (126, 216), and in certain strains of S. mutans (185a). Interspecies and intergeneric transformation has also been reported (22, 58, 182, 186, 233).

Plasmid transformation was first reported by LeBlanc and co-workers (130, 133), who introduced pAMβ1(Em') from S. faecalis DS5 into group H (S. sanguis strain Challis) and group F streptococci. pAMα1(Tc') from DS5 would not transform. Several other plasmids from different species and genera have now been shown to transform the Challis strain (13, 24, 146, 155), as well as S. pneumoniae (8, 199, 205, 211), and two streptococcal plasmids (pAMα1 and pAMβ7) have been shown to transform B. subtilis (42, 241).

Using an approach originally used in E. coli (120), Macrina and co-workers (146) were able to transform strain Challis with small cryptic plasmids from Streptococcus faecalis (formerly S. mutans). This was done by transforming with an excess of the S. fecalis strain V380 cryptic plasmids pVA380 and pVA380-1 mixed with pVA1 (a small deleted form of pAMβ1); a significant number of the selected Em' transformants also contained one of the unselected plasmids.

Transformation in S. sanguis and S. pneumoniae is dependent on a competent state that is acquired for a short period of time at an optimum cell density during logarithmic growth (63, 183, 217). Unlike B. subtilis, where competence is associated with a minor biosynthetically inactive fraction of the population (65), streptococcal competence involves the entire population (17, 215). A small protein called competence factor is involved in triggering a synchronous competence response (183, 216, 217). Recent studies with both S. pneumoniae (173) and S. sanguis (191) have shown that synthesis of a number of specific proteins is induced during generation of the competent state.

It is believed that in the course of transformation, only a single strand of DNA enters a cell; the complementary strand is degraded at the cell surface (126). Lacks (125) has shown that in S. pneumoniae covalently closed circular DNA is nicked during binding to the cell surface and is converted to single strands during uptake. Because of a presumed absence of internal homology, there is probably a problem in circularization; thus, it is not surprising that some plas-
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mids have been shown to undergo deletions readily in the course of establishing residency in the new host (13, 42, 133, 141, 142, 205). In a few cases, some specificity in the nature of such deletions has been suggested (14, 142). Consistent with this notation is the finding that oligomeric forms of plasmids transform S. sanguis and S. pneumoniae more efficiently than monomeric forms (140, 199, 200). Indeed, dimeric molecules transform with single-hit kinetics, whereas monomeric forms exhibit double-hit kinetics; i.e., the uptake of two molecules is necessary for successful transformation. The additional homology of dimers and recombination between two monomers appear to facilitate the circularization process. Additional support for this hypothesis comes from observations (8, 200) that plasmid DNA linearized by a single restriction enzyme failed to transform S. pneumoniae unless it was mixed with homologous DNA linearized with a different restriction enzyme. Furthermore, plasmid DNA linearized by cleavages at random sites with S1 nuclease maintained its transformability (8, 200). It is interesting that in the case of B. subtilis transformation, monomeric plasmid forms appear to be completely inactive, with virtually all transformation being due to oligomeric forms (26, 174).

DRUG RESISTANCE AND TRANSPONS

In the last 15 to 20 years, isolates of streptococci resistant to erythromycin, chloramphenicol, aminoglycosides, or tetracycline or combinations of these drugs have become increasingly common (78, 159). Although it is clear (Table 1) that resistance is commonly plasmid borne, examples of resistance not related to plasmids have also been observed (see above). The frequency of resistant strains depends on the drug, the species, and the geographical location. In Japan, the majority of S. pyogenes isolates are now resistant to one or more drugs (168, 169, 175, 176, 223), and more than 50% (primarily type T-12) have been reported (175, 176) to be resistant to tetracycline, erythromycin, and chloramphenicol simultaneously. Significant but lower frequencies have been reported in other countries (15, 20, 56, 60, 62, 78). Tetracycline resistance is extremely common among group B and D species throughout the world (4, 7, 24, 73, 77, 114, 171, 189). A variety of oral (viridans) isolates exhibit tetracycline resistance, and highly elevated frequencies have appeared in patients receiving antibiotic therapy (135). Of particular concern in recent years has been the appearance of multiple drug resistance in S. pneumoniae, with some strains showing resistance to more than eight different drugs (27, 113, 229).

Streptococcal resistance to aminoglycosides and chloramphenicol is mediated by drug-modifying enzymes (51, 54, 106, 122, 170, 208), much like the case in other genera (59). Erythromycin (MLS) resistance appears to be due to an N6 dimethylation of an adenosine residue in 23S ribosomal ribonucleic acid, and streptococci resemble resistant streptococci in this regard (127, 230). In some cases resistance is inducible (109, 230); a relatively short (15- to 60-min) exposure to a low concentration of erythromycin (e.g., 0.01 μg/ml) results in a high level of resistance (minimal inhibitory concentration, >1 mg/ml).

It is interesting that the great majority of streptococcal plasmids which reportedly determine erythromycin resistance have molecular weights of 15×10^6 to 20×10^6 (Table 1). Two of these plasmids (pAMβ1 and pAC1) are more than 90% homologous (240), and comparisons with other plasmids have revealed common restriction fragments (98). Three other MLS plasmids were also shown to have extensive homology (74). Weisblum and co-workers (231) have demonstrated significant homology between MLS resistance determinants of different streptococcal species and staphylococci. It will be interesting to determine the extent to which erythromycin resistance plasmids are related with regard to replication and compatibility properties.

Many strains of S. pyogenes exhibit a zonal pattern of resistance to lincomycin (61, 152, 155). This is characterized by growth only at subinhibitory concentrations (<0.06 μg/ml) and at concentrations between 60 and 250 μg/ml, but not at intermediate or higher concentrations. It has been reported (155) that lincomycin at concentrations between 60 and 200 μg/ml (i.e., zonal concentrations) induces lincomycin resistance in situ, whereas at lower concentrations resistance remains repressed. Higher concentrations do not induce resistance, presumably due to the overwhelming inhibition of protein synthesis. Low concentrations of erythromycin (e.g., 0.05 μg/ml) induce resistance to increased levels of erythromycin and intermediate concentrations of lincomycin. The absence of resistance to streptogramin type B antibiotics (155) distinguishes these strains from the strains having the more conventional MLS phenotype. In at least one case, zonal resistance was shown to be plasmid related (155).

Considering the heavy use of the penicillins over many years, it is surprising that streptococci have remained essentially sensitive to these drugs. Although there have been reports of relatively low levels of resistance among S.
pneumoniae (62), S. agalactiae (201), and the viridans group (21, 78), there have been no reports of beta-lactamase production associated with resistance. In view of the ease with which streptococci have been able to acquire resistance to other drugs and the likelihood that staphylococci and streptococci exchange genetic information in nature (see above), there is no obvious reason why beta-lactamase genes should be prevented from entering streptococci. Therefore, the absence of this phenotype may reflect an inability to express or excrete beta-lactamase; alternatively, expression of the enzyme could be lethal to the cell. Although this question could be studied easily by introducing a staphylococcal beta-lactamase plasmid into streptococci, this approach might be considered unwarranted on ethical grounds.

With regard to the genetics of drug resistance at the molecular level, much of the published work has dealt with systems in S. faecalis. These systems are discussed below.

**Amplifiable Tetracycline Resistance Determinant on pAMa1**

Originally identified in S. faecalis DS5 (39, 41), pAMa1 is a nonconjugative, 6-Mdal, multicopy plasmid that determines tetracycline resistance. It has been observed that growth of strain DS5 in the presence of tetracycline resulted in an enlargement of the plasmid; this phenomenon was analyzed further after pAMa1 was mobilized into a JH2-2 host, where it could be studied in the absence of other plasmids (38, 40, 236–238). The tetracycline resistance determinant was found to be located on a 2.65-Mdal segment of DNA which was defined by two directly repeated 0.38-kilobase sequences (designated RS1). Cells grown for a number of generations in the presence of tetracycline exhibited increased levels of resistance and harbored plasmid DNA consisting of a heterogeneous population of molecules containing differing numbers of tandem repeats of the 2.65-Mdal segment. The amplification phenomenon was reversible; in addition, tetracycline-sensitive variants with a 2.65-Mdal deletion appeared at a frequency of about 1% during growth in the absence of drug. Neither amplification nor deletions occurred in a Rec− derivative (239) of JH2-2 (238), and three models (all based on recombination between the direct repeats) have been suggested (39, 237) as the basis for amplification (Fig. 6).

Interestingly, it has been observed (236) that as pAMa1 gets larger, the number of copies of the plasmid decreases; the mass of plasmid DNA calculated as a percentage of the chromosomal mass remains the same in amplified and unamplified cells. The reason for this is not clear; a copy number control mechanism which is sensitive to total plasmid mass appears to be involved (see below).

Although the amplifiable tet element with its direct repeats resembles a transposon, its transposition to other replicons has never been observed. Because pAMa1 is mobilized so easily by conjugal plasmids, screening for transposition to the latter is difficult. In addition, insofar as the level of tetracycline resistance is probably determined by the sum of the determinants, transposition from a multicopy plasmid to a single-copy conjugative plasmid might result in a level of tetracycline resistance too low to resolve easily.

Amplification phenomena similar to the amplification of pAMa1 have been found in certain large R-plasmids in Proteus mirabilis (196, 197) and E. coli (28, 160, 165, 178). In these, the amplifiable determinants are also flanked by direct repeats, and in certain cases the flanking repeats represent the insertion sequence IS1 (28, 108, 165, 190).

**Resistance Transposons**

Two resistance transposons have been characterized in S. faecalis. Both of these (Tn916 [79, 80] and Tn917 [37, 218, 219]) were identified originally in the clinical isolate DS16. Tn916 (10 Mdal), which confers tetracycline resistance, is chromosome borne and, as discussed above, appears to have fertility properties (79, 80). This transposon has been shown to insert into several sites in different conjugative hemolysin plasmids (pAD1, pAM-y1, and pOB1) at a frequency of $10^{-6}$ to $10^{-7}$. Efforts to reveal inverted repeats in this element on the basis of self-annealing studies with electron microscopy have been unsuccessful (A. Franke, personal communication).

Some insertions of Tn916 into pAD1 result in the inactivation of hemolysin expression whereas others result in a hyperexpression of hemolysin, giving rise to zones of hemolysin three to four times the normal diameter on horse blood agar (79, 80). With certain insertions, the appearance of hyperexpression of hemolysin is stimulated by the presence of tetracycline in the blood agar (A. Franke, unpublished data); the absence of the drug results in nonhemolytic colonies. In the latter types, if a blood agar plate lacking tetracycline with mature colonies is allowed to incubate (age) for an additional few days, a wave of hemolysin expression appears to spread through the plate; the expression of one colony seems to trigger the expression of nearby
Fig. 6. Models for the generation of plasmid DNA molecules with deletions or repeats of the tetracycline resistance determinant. In Ia through Ic, an intramolecular recombinational event results in the looping out of a small 2.65-Mdal circle, which then recombines with an intact molecule of pAMal, resulting in a duplication. IIa through IIb show an uneven recombinational event between RS1 sequences on the daughter strands of a partially replicated plasmid. Depending on the nature of events that follow, this structure could give rise to a dimeric structure (IIIb) or to two independent molecules, one of which contains a duplication and one of which contains a deletion. In the case where a dimeric structure is generated, a subsequent recombinational event between two RS1 sequences (dashed lines in IIIb) would be required to generate two independent molecules. The third model (IIIa and IIIb) involves an uneven recombinational event between the RS1 sequences of two independent molecules, resulting in a dimeric structure. A subsequent recombinational event would generate two independent molecules, one with a duplication and the other with a deletion. Additional amplification could arise by repetitions of these events; however, the process would no longer be dependent on RS1 sequences for homologous recombination.

colonies. Similar observations have been made by LeBlanc (personal communication) in connection with a tetracycline resistance element originating in S. faecalis strain JH1, which inserts into the hemolysin plasmid pH2. (In all cases, the drug-related hemolysin expression phenomena were observed in a JH2-2 host.) The basis of hyperexpression of hemolysin and its stimulation in some cases by tetracycline remains a mystery. Thus far, all Tn916 insertions which influence hemolysin expression map in a region around seven o'clock on pAD1 (Fig. 1).

Tn917 is a 3.3-Mdal transposon that carries an inducible erythromycin (MLS) resistance determinant. Originally identified on the nonconjugative pAD2 plasmid of strain DS16, this transposon has been shown to transpose to co-resident pAD1 at a frequency of $10^{-6}$ (219). Tn917 is flanked by 0.28-kilobase repeated sequences which are inverted with respect to each other (219). Insertion occurs into multiple sites in pAD1 and, in some cases, results in inactivation of hemolysin expression (P. Tomich, C. Gawron-Burke, A. Franke, F. An, and D. Clewell, submitted for publication). These sites map in EcoRI fragment H (Fig. 1). Unlike Tn916 insertions, Tn917 insertions in pAD1 so far have not been observed to give rise to hyperexpression of hemolysin.

Tn917 has the interesting feature that trans-
position is inducible upon exposure of cells to low concentrations of erythromycin (less than 0.5 \mu g/ml) (218, 219). A 4-h exposure of strain DS16 cells to 0.5 \mu g of erythromycin per ml results in a greater than a 10-fold increase in pAD1 molecules with newly acquired Tn917 insertions. Studies have shown that transposition involves a pAD1::pAD2 cointegrated structure which forms during the first 20 to 30 min of induction; resolution of the cointegrated structure into pAD1::Tn917 and pAD2 takes longer, requiring as much as 30 min or more (219; Tomich et al., submitted for publication). If strain DS16 cells are mated with strain JH2-2 (plasmid-free) recipients after 30 min of induction with erythromycin, followed by washing the cells free of drug, the majority (>90%) of the erythromycin-resistant transconjugants contain a cointegrated molecule which remains stable in the JH2-2 host. Such structures have been observed to have two Tn917 elements situated at the two junction points of the plasmids (Tomich et al., submitted for publication). Evidently, the JH2-2 host lacks a function which was present in strain DS16 and facilitates the resolution step. In general, the mechanism of transposition appears to be entirely consistent with models proposed by Shapiro (202) and Arthur and Sherratt (5), which were based heavily on information gained from studies of the behavior of Tn3 (96). It is conceivable that the determinants of MLS resistance (possibly encoding an enzyme which methylates an adenine residue in 23S ribosomal ribonucleic acid) and a transposase are linked together on a single operon or are controlled by a common regulatory function. In view of recent data reported by Horinouchi and Weisblum (103) and Shivakumar et al. (203) suggesting that MLS resistance in staphylococci is controlled by posttranscriptional processes, the possibility that such a control process exists for Tn917 resistance and transposition functions remains open.

It is noteworthy that the only other transposons that have been identified in gram-positive bacteria are Tn551 and Tn554, both of which occur in S. aureus (177, 184, 185, 187).

PLASMID MAINTENANCE AND INCOMPATIBILITY

Very little is known about plasmid incompatibility and replication (maintenance) functions in streptococci. Although numerous plasmids have been identified, attempts to categorize them into specific compatibility groups have been limited by the lack of convenient combinations of selectable plasmid markers. In the case of the R-plasmids, many bear only MLS resistance markers. In other cases, conjugative plasmids harbor hemolysin determinants or bacteriocin determinants or both, which are not amenable to positive selection. It is likely that, as in the gram-negative species, many compatibility groups will be found eventually. This is certainly supported by the observation that some strains of S. faecalis harbor as many as five plasmids (e.g., strains DS5 and 39-5) (Yagi, Brown, and Clewell, unpublished data). Six or more plasmids have been observed in certain group N strains (129, 136).

Evidence recently reported by Romero et al. (195) placed members of a collection of S. faecalis R-plasmids into three groups. (It takes at least two plasmids to define a group.) Recent studies (Brown and Clewell, unpublished data) have also shown that pAD1 and pAM\gamma 1 are incompatible; that is, these plasmids are members of the same group. Although these two plasmids both determine hemolysins, the construction of derivatives with different resistance transposons allowed compatibility testing. Two other hemolysin plasmids (pOB1 and pPD5) tested by this approach were found to be compatible with pAD1 and pAM\gamma 1 (Brown and Clewell, unpublished data).

As in other genera, in streptococci there are examples of multicopy plasmids and plasmids that are present to the extent of only one to two copies per chromosomal genome equivalent. Usually, the smaller plasmids are present as multicoipies, although there are exceptions, such as the 4.5-Mdal pAM77 plasmid in S. sanguis (241) and the 2.0-Mdal pDP1 in S. pneumoniae (210), which occurs at only one to two copies per chromosomal genome equivalent. Macrina et al. (141, 142) have constructed derivatives of pAM\beta 1 in S. sanguis which, in contrast to the parent form, are present as multicoipes. This suggests that pAM\beta 1 has a negatively acting copy number control function which is missing (deleted?) or defective in the derivatives.

An interesting copy number control process has been found in the tetracycline resistance gene amplification of pAM\alpha 1, a phenomenon whereby the mass of plasmid DNA taken as a percentage of chromosomal mass remains constant (see above). Although the basis of this phenomenon is unknown, the possible interpretations are as follows. (i) Because of a limited number of replication sites (e.g., one to two sites for the 10 copies per chromosomal genome equivalent in the unamplified state), the initiation of a round of replication requires that the previous round be completed. Thus, assuming that completion of a round of replication re-
quires at least several minutes, replication of the larger molecules occupies a greater fraction of the division cycle, and initiation of new rounds is less frequent (236). Fewer initiations result in fewer copies. (ii) The amplified segment on pAMa1 includes a determinant that encodes a function which affects initiation negatively. The increased gene dosage arising because of amplification results in fewer copies.

A few MLS resistance plasmids identified originally in S. pyogenes have unusually long inverted repeats; together these repeats occupy 40 to 80% of the plasmid (12, 19). In the case of pSM19035, which has 80% of its DNA corresponding to such repeats, a deletion has been obtained in S. sanguis strain Challis (13) where ends of the long repeats appear to be fused (14). This deletion is designated pDB102, and less than 10% of the molecule corresponds to DNA that is not repeated. This small segment (0.93 Mdal) contains the MLS resistance determinant (10). Since there is little room for further genetic information in this region, it has been suggested (14) that functions related to replication are on the repeated DNA sequences. Therefore, replication genes may be present in duplicate and may be arranged in reverse orientation with respect to each other. It also follows that there may be two replication origins. Analyses of the replication of pSM19035 should provide information on whether there are in fact two origins and, also, whether replication is unidirectional or bidirectional. It will be interesting to see whether analyses of replication also shed some light on why pSM19035 stably maintains such extraordinarily long duplicate sequences.

**MOLECULAR CLONING**

The use of recombinant DNA technology in molecular studies of streptococcal DNA should greatly facilitate genetic and biochemical analyses, including investigations of the regulation and control of gene expression. Whereas standard E. coli vectors have already been useful in analyses of S. mutans DNA (94; R. Curtiss III, personal communication), there are certain problems which can be anticipated in the study of gene products which are excreted or relate to the bacterial surface. This is due primarily to the extensive differences between gram-positive and gram-negative bacteria with regard to the cell membrane-wall complex. Such differences might affect the passage of proteins through this barrier. Since in streptococci there is a great deal of interest in factors related to pathogenicity, many of which deal with substances that are excreted (e.g., toxins) or are located on the surface (e.g., attachment substances, surface antigens, etc.), the availability of gram-positive vector systems would be advantageous. Recently developed systems in B. subtilis (66, 138) may be valuable for such studies; however, recently progress has been made in the development of streptococcal systems in which the transformable S. sanguis strain Challis is used (10, 11, 141, 155). Some plasmid derivatives which have potential as cloning vectors are listed in Table 2. Some of these derivatives (e.g., pVA1 and pVA677) are unstable and are lost spontaneously at frequencies as high as 90% if the cells are grown at 42°C (141). (In certain investigations, this instability and temperature sensitivity could be useful.) DNA segments containing MLS resistance determinants have been recombined with small cryptic plasmids from S. mutans (S. ferus), resulting in multicopy chimeras which are very stable (10, 141). Using the MLS resistance determinant of pVA1 and the cryptic plasmid pVA380-1, Macrina et al. (141) have constructed and physically mapped several chimeric plasmids (pVA690, pVA736, and pVA738), one of which (pVA736) was used successfully to clone the chloramphenicol resistance determinant of the R-plasmid pIP501, as well as chromosomal sequences from S. mutans strain 6715-13. Similar studies have been reported by Behnke and Ferretti (10) and Malke et al. (153a).

A problem which arises in molecular cloning experiments with S. sanguis (and also B. subtilis) concerns the generation of unwanted deletions during the process of transformation (see above section on Transformation). Recent efforts to overcome this problem involve the transformation of strains which already harbor a plasmid consisting of some component of the vector plasmid (47a), so that recombinational events with the entering vector plasmid facilitate circularization (F. L. Macrina and W. Guild, personal communication).

A recent report by Hansen et al. (94) described the cloning of the small cryptic plasmid pVA318 from S. mutans into the pBR322 vector system of E. coli. An examination of minicells showed that the S. mutans plasmid encodes a 20,000-dalton protein; the function of this protein is not known. In addition, Burdett (personal communication) recently cloned into E. coli pAMa1, pMV158, and pMV163, as well as a chromosomal tet determinant from S. agalactiae B109, and she observed expression of resistance.

**CONCLUSIONS**

Information on the nature of plasmids and their exchange in streptococci is accumulating...
Table 2. Potential cloning vectors in S. sanguis strain Challis

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mol wt (×10^6)</th>
<th>Relevant marker(s)</th>
<th>Parent plasmid(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVA1</td>
<td>7.3</td>
<td>Em'</td>
<td>pAM81</td>
<td>142</td>
</tr>
<tr>
<td>pVA677</td>
<td>5.0</td>
<td>Em'</td>
<td>pVA1</td>
<td>141</td>
</tr>
<tr>
<td>pVA380-1</td>
<td>2.9</td>
<td>Cryptic</td>
<td>Same (from S. ferus)</td>
<td>141, 146</td>
</tr>
<tr>
<td>pVA680</td>
<td>5.5</td>
<td>Em'</td>
<td>pVA380-1, pVA1</td>
<td>141</td>
</tr>
<tr>
<td>pVA736</td>
<td>5.1</td>
<td>Em'</td>
<td>pVA380-1, pVA1</td>
<td>141</td>
</tr>
<tr>
<td>pVA738</td>
<td>3.7</td>
<td>Em'</td>
<td>pVA380-1, pVA1</td>
<td>141</td>
</tr>
<tr>
<td>pVA743</td>
<td>9.3</td>
<td>Em', Cm'</td>
<td>pVA736, pIP501</td>
<td>141</td>
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<td>pDB101</td>
<td>12</td>
<td>Em'</td>
<td>pSM19035</td>
<td>11</td>
</tr>
<tr>
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<td>4.9</td>
<td>Em'</td>
<td>pVA318, pSM19035</td>
<td>10</td>
</tr>
<tr>
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<td>Em', Cm'</td>
<td>pIP501</td>
<td>11a</td>
</tr>
<tr>
<td>pGB307</td>
<td>3.6</td>
<td>Em'</td>
<td>pGB301</td>
<td>11a</td>
</tr>
<tr>
<td>pSM10</td>
<td>5.4</td>
<td>Em'</td>
<td>pSM10419</td>
<td>153a</td>
</tr>
<tr>
<td>pC221</td>
<td>2.9</td>
<td>Cm'</td>
<td>Same (from S. aureus)</td>
<td>153a</td>
</tr>
<tr>
<td>pSM10221</td>
<td>8.3</td>
<td>Em', Cm'</td>
<td>pSM10, pC221</td>
<td>153a</td>
</tr>
<tr>
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<td>Tc'</td>
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<tr>
<td>pMV163</td>
<td>3.6</td>
<td>Tc'</td>
<td>Same (from S. agalactiae)</td>
<td>24</td>
</tr>
<tr>
<td>pAM77</td>
<td>4.5</td>
<td>Em'</td>
<td>Same (from S. sanguis)</td>
<td>241</td>
</tr>
<tr>
<td>pAM510</td>
<td>6.8</td>
<td>Em', Sm'</td>
<td>pAD2, pVA736</td>
<td>Yagi, unpublished data</td>
</tr>
<tr>
<td>pAM511</td>
<td>7.5</td>
<td>Em', Km'</td>
<td>pAD2, pVA736</td>
<td>Yagi, unpublished data</td>
</tr>
</tbody>
</table>

rapidly. Plasmids confer a variety of different properties, and many plasmids have the ability to conjugate or to be mobilized by coressident conjugative plasmids. Some of these plasmids have broad host ranges and can become established in other genera (e.g., Lactobacillus, Staphylococcus, or Bacillus) by conjugation or transformation. (For a discussion which includes conjugative plasmids originating in other gram-positive genera, see reference 31). In the case of certain conjugative plasmids in S. faecalis, a novel signaling mechanism has been found, whereby recipient strains excrete sex pheromones which induce donors to mate. Clear evidence for sex pheromones in other species of bacteria has not been reported, although sex-related chemotactic factors have been suggested in E. coli (46) and Salmonella typhimurium (16). (Sex pheromones are well known in higher organisms [119, 159].) For other streptococcal plasmids, laboratory mating requires forced contact (e.g., on filter membranes). In nature, such contact may arise as bacteria crowd and layer upon each other on tissue surfaces, such as in the mouth or gut. For example, the bacteria in dental plaque and tongue coatings may exist in a state conducive to conjugal transfer.

Chromosome-borne resistance determinants which transfer in the absence of conjugative plasmids appear to be common in streptococci and may represent a new class of elements typified by Tn916. Although such elements have not been identified in other genera, recently there have been indications that transferrable nonplasmid elements may indeed exist in Clostridium difficile (209) and Bacteroides fragilis (160a, 214a). A variety of different genetic determinants, including determinants relating to pathogenicity, may be found on such elements.

Because of their prevalence in the gut and their frequent appearance in the oral cavity, it is possible that S. faecalis strains (the "E. coli" of the gram-positive bacteria?) may serve as reservoirs of genetic information available for passage to other streptococci and even other genera by conjugal processes. These organisms have an abundance of plasmids and can readily distribute information from both plasmids and chromosomes.

The recent advances in the development of cloning systems should contribute significantly to the ease and speed with which genetic and biochemical analyses of streptococci and related bacteria can be carried out. In turn, our understanding of the basic features of drug resistance, pathogenicity, and gene transfer should progress rapidly. The use of these systems for commercial purposes or medical purposes or both can also be anticipated.

ACKNOWLEDGMENTS

I thank my numerous colleagues for unpublished and published information provided so that this review could be as up to date as possible. I also thank C. Gawron-Burke for her careful reading of the manuscript and her constructive comments and Y. Yagi, P. Tomich, R. Craig, C. Gawron-Burke, Y. Ike, A. Franke, D. Schaberg, R. Kessler, D. Lopatin, G. Dunny, B. Brown, and F. An for helpful discussions.

Work in my laboratory was supported by Public...
Health Service grants DE02731 and AI10318 from the National Institutes of Health.

LITERATURE CITED


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