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Host-Vector System for Integration of Recombinant DNA into Chromosomes of Transformable and Nontransformable Streptococci

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We describe a genetic system in which transformation of *Streptococcus pneumoniae* and *Streptococcus sanguis* was used to insert recombinant DNA into the conjugative chromosomal element Ω(*cat tetM*) 6001 (Ω6001). The element containing the recombinant DNA was then transferred by conjugation to the chromosome of transformable and nontransformable streptococci. When *Escherichia coli* plasmid pDP36 was used as donor in transformation, it was capable of inserting 5.9 kilobases of heterologous DNA into the chromosome of competent streptococcal strains carrying Ω6001; the transformants were scored for erythromycin resistance. Genetic analysis showed that in a fraction of the erythromycin-resistant transformants the integration via flanking homology of the heterologous DNA caused inactivation of the *tetM* gene of Ω6001. By analyzing the stability of the resistance markers, we found that stable integration of heterologous DNA was achieved only in the erythromycin-resistant, tetracycline-sensitive transformants. It was possible to detect conjugal transfer of the heterologous sequences from stable transformants to strains of *S. pneumoniae*, *S. sanguis*, *Streptococcus pyogenes*, and *Streptococcus faecalis*. The Ω6001-pDP36 host-vector system opens new possibilities for gene transfer in streptococci. By this method cloned streptococcal DNA (possibly mutagenized in vitro) can be returned to the original host, greatly facilitating complementation tests and fine physiological studies.

Recombinant DNA techniques allow cloning of genes from a variety of organisms. One of the major problems of the cloning approach in biological research is how to return the cloned DNA to the original host, where gene functions can be better studied. Suitable gene transfer methods need to be developed, which will allow the study of a cloned gene (possibly mutagenized in vitro) under "physiological" conditions in the original host.

Plasmid transfer techniques were developed (15) and used (13) to return cloned streptococcal DNA to its original host. However problems may arise when trying to perform complementation tests or fine physiological studies with genes carried on plasmids rather than on the bacterial chromosome. These problems are mainly due to plasmid instability and to the fact that plasmid copy number can affect the physiology of gene expression, adding to the analysis of an already rather complex situation a variable that is sometimes difficult to evaluate.

In this paper we describe a system that allows stable integration of recombinant DNA molecules into chromosomes of transformable and nontransformable streptococci. By transformation, recombinant DNA is first inserted into a streptococcal conjugative element that is already integrated into the chromosome of a competent strain of *Streptococcus pneumoniae* or *Streptococcus sanguis*. The recombinant DNA is then transferred by conjugation to the chromosome of other streptococci. This approach is based on the conjugation properties of certain chromosomal elements found in clinical isolates of streptococci, some of which have been defined as "conjugative transposons" (1, 2, 4), and on the possibility of achieving stable and efficient integration of heterologous DNA during transformation (10).

Bacterial strains are listed in Table 1. Pneumococcal strains were grown in CAT medium (9), whereas brain heart infusion (Difco Laboratories, Detroit, Mich.) was used for all other bacteria. Agar (1.5%) was added to either broth to obtain solid medium. All bacterial cultures were incubated at 37°C.

Insertion vector pDP36. pDP36, a 19.3-kilobase (kb) *Escherichia coli* plasmid, was used to insert 5.9 kb of heterologous DNA into the chromosome of a streptococcal host carrying the conjugative chromosomal element Ω6001 (Fig. 1). pDP36 was obtained during the cloning and restriction mapping of Ω6001; it was described by Vijayakumar and colleagues (20, 21). In pDP36 the 14.4-kb *Kpn*I fragment of Ω6001 containing the *tetM* gene is interrupted by pVA891 (7) and has a deletion of 1.1 kb where the pVA891 heterologous sequences are inserted (Fig. 1). A key feature of our system is that this deletion inactivates the tetracycline resistance (Tc^r) marker (20, 21). pVA891 sequences provide pDP36 with an origin of replication, a chloramphenicol resistance (Cm^r) marker that is expressed in *E. coli*, and an erythromycin resistance (Em^r) marker that can be scored for in streptococci. The pVA891 sequences (heterologous DNA) and the site of the 1.1-kb deletion are depicted in Fig. 1, together with a restriction map of pDP36 (Pozzi and Guild, unpublished results). In pDP36 sequences of Ω6001 are on both sides of pVA891 (Fig. 1); they provide the flanking homology that allows stable integration of the heterologous DNA (pVA891) into the chromosome during transformation of a competent streptococcus containing the conjugative element Ω6001, according to the model discussed previously (10).

Chromosomal integration during transformation. pDP36 purified by CsCl-ethidium bromide density gradient ultracentrifugation was used to transform *S. pneumoniae* DP1322 and *S. sanguis* GP201, both of which contain the chromosomal element Ω6001. The plasmid preparation obtained from strain GP307 (*E. coli* C600, $RecA^+$) contained different supercoiled forms of pDP36 in a ratio of approximately two

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TABLE 1. Bacterial strains

Strain	Relevant properties[a]	Origin and source or reference
S. pneumoniae		
DP1322	Ω(*cat tetM*)	Carries the conjugative chromosomal element Ω6001 (14, 20, 21)
GP88	Ω(*cat* Δ*tetM*::pVA891)	Transformation of DP1322 with pDP36 (this work)
DP1002	*nov-1*	3
DP1004	*str-1*	3
S. sanguis Challis		
V288	Standard recipient	8
V481	Rif^r	Rif^r derivative of V288 (F. Macrina, Virginia Commonwealth University)
GP204	*str-204*	Spontaneous mutant of V288 resistant to high levels of streptomycin (this work)
GP201	*str-204* Ω(*cat tetM*)	Conjugation of Ω6001 from DP1322 to GP204
GP202	*str-204* Ω(*cat* Δ*tetM*::pVA891)	Transformation of GP201 with pDP36
S. pyogenes D471	Str^r	V. Fischetti, The Rockefeller University
S. faecalis JH2-2	Rif^r Fus^r	5
S. faecium ATCC 9790 LM1	Rif^r Fus^r	Spontaneous mutant of ATCC 9790 (L. Marri, Temple University)
S. mutans GS5-Str	Str^r	L. Daneo-Moore, Temple University
E. coli GP307	pDP36	This work

[a] Ω(*cat tetM*), Chromosomal element with genes for resistance to chloramphenicol and tetracycline; Ω(*cat* Δ*tetM*::pVA891), chromosomal element with a *tetM* gene with a deletion and the insertion of plasmid pVA891 (*ermB*); *nov-1 str-1 str-204*, chromosomal point mutations conferring resistance to novobiocin and streptomycin; Rif^r, Fus^r, and Str^r, resistance to rifampin, fusidic acid, and streptomycin, respectively.

monomers per dimer, as judged by fluorescence on agarose gels (data not shown). Preparation of frozen competent cells of *S. pneumoniae* and *S. sanguis* and transformation procedures were essentially as already described (3, 6, 11a, 12, 14).

pDP36 very efficiently transformed DP1322 to erythromycin resistance, inserting the heterologous sequences of pVA891 into the chromosomal element Ω6001; 0.1 μg of plasmid DNA per ml of competent cells yielded 5.0×10^5 CFU of transformants per ml (competent cells were at a concentration of 6.2×10^7 CFU/ml), and transforming activity was still detectable when pDP36 was at a concentration of 1 pg/ml. The dose response for transformation of DP1322 with pDP36 showed one-hit kinetics (data not shown). Genetic analysis was performed on 300 erythromycin-resistant transformants, by toothpick transfer of colonies from selection plates to blood agar plates containing antibiotics (tetracycline, 2 μg/ml; chloramphenicol 5 μg/ml; erythromycin, 2 μg/ml). All 300 were resistant to erythromycin and chloramphenicol, whereas 13% had lost the tetracycline resistance. Five tetracycline-susceptible and five tetracycline-resistant transformants from this experiment were also

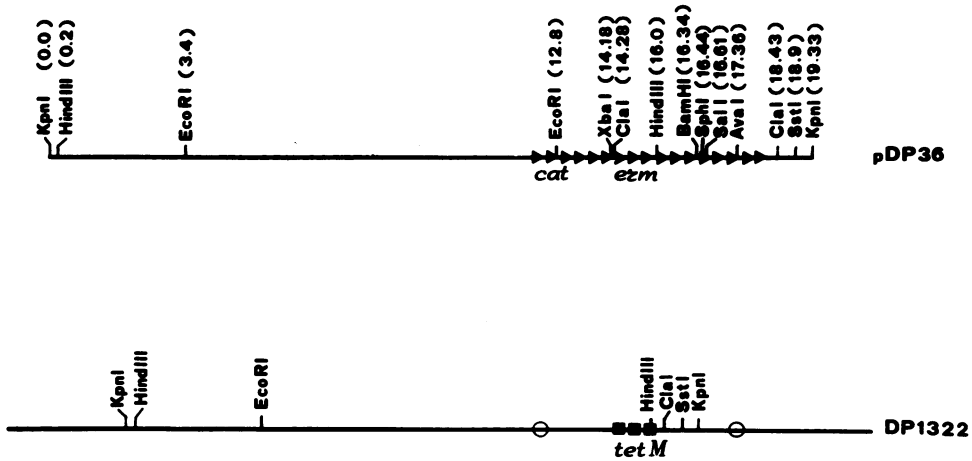

FIG. 1. Restriction map of pDP36 and of the homologous region of the Ω6001 element in the chromosome of DP1322. *S. pneumoniae* DP1322 was used as the recipient in transformation; it carries the element Ω6001 integrated into the chromosome. The 14.4-kb *Kpn*I fragment of Ω6001 containing the *tetM* gene is present in pDP36, where its sequences are interrupted by pVA891 (▶): in pDP36, at the site of insertion of pVA891, there is a deletion of 1.1 kb in the Ω6001 sequences (■). pVA891 sequences (▶) present in pDP36 are heterologous with respect to Ω6001. During transformation the integration of the heterologous DNA (▶) into the 5.0-kb *Hin*cII (○) fragment containing the *tetM* gene produces a 1.1-kb deletion (■) that inactivates the tetracycline resistance marker. pDP36 does not carry sequences homologous to the pneumococcal chromosome, but only to Ω6001. In fact, in the chromosome of DP1322, Ω6001 sequences extend about 30.2 kb to the left and 20.9 kb to the right of the 14.4-kb *Kpn*I fragment (20).

assayed for the stability of their resistance markers. After growth in broth without selection for about 50 generations, cells were plated; 100 CFU were tested for resistance to tetracycline and erythromycin. The tetracycline-susceptible (Tc^s) transformants showed a stable Em^r Tc^s phenotype, whereas the Tc^r transformants showed loss of either resistance marker (the Em^s Tc^s phenotype was never found).

The chromosomal element Ω6001 was also transferred by conjugation from DP1322 to GP204, a spontaneous streptomycin-resistant mutant of *S. sanguis* Challis strain V288 (8), to yield GP201 (Table 2). When the same preparation of pDP36 was used to transform *S. sanguis* GP201, 0.01 μg of DNA per ml of competent cells yielded 8.8×10^4 CFU of Em^r transformants per ml. In this case the resistance to tetracycline was lost by 76% of the 300 Em^r transformants analyzed. As observed for *S. pneumoniae*. Em^r Tc^s transformants were stable, whereas Em^r Tc^r transformants lost either marker when grown without selection.

The presence of dimeric forms of pDP36 in the preparation used for transformation can account for the occurrence of the Em^r Tc^r doubly resistant transformants, whereas the instability of the resistance markers in such transformants can be explained with the formation of direct repeats during the integration of dimers as described previously (10, 18, 19). Two hypothesis that can explain why the same preparation of pDP36 yields a higher fraction of Em^r Tc^s transformants are currently under investigation. There may be a higher frequency of recombination between direct repeats in *S. sanguis*, leading to an increased instability of the Tc^r marker; or in *S. sanguis* the processing of incoming DNA during transformation may be such that shorter strands end up recombining with the chromosome. It should be pointed out that, for the purpose of this work, the interesting feature of this system is that the inactivation of the Tc^r marker means stable integration of heterologous DNA into the conjugative chromosomal element.

Conjugation experiments. The chromosomal element Ω6001 containing the heterologous DNA integrated into the *tetM* gene was transferred by conjugation to transformable and nontransformable streptococci (Table 2). *S. pneumoniae* GP88, isolated after transformation of DP1322 with pDP36, was used as donor in conjugation. GP88 is one of the stable transformants (Em^r Tc^s) that carry the sequences of pVA891 integrated into the chromosomal element Ω6001. GP202 is the *S. sanguis* equivalent of GP88, is Em^r Tc^s, and was isolated after transformation of GP201 with pDP36; GP202 was also used as a donor. Conjugation experiments were carried out by filter mating as described in detail by Smith and Guild (16). The chromosomal element carrying the heterologous sequences was transferred to *S. pneumoniae*, *S. sanguis*, *Streptococcus faecalis*, and *Streptococcus pyogenes*, when *S. pneumoniae* GP88 was the donor, whereas successful transfer was found only to *S. pneumoniae* when the donor was *S. sanguis* GP202 (Table 2). Upon genetic analysis 100% of the transconjugants tested, from all the positive conjugation experiments, were Em^r Tc^s Cm^r.

TABLE 2. Conjugation experiments

Donor	Recipient	Selection[a]	Transfer frequency[b]
S. pneumoniae			
GP88	*S. faecalis* JH2-2	Em^r Rif^r Fus^r	3.8×10^{-6}
GP88	*S. pyogenes* D471	Em^r Str^r	6.7×10^{-6}
GP88	*S. sanguis* GP204	Em^r Str^r	1.3×10^{-7}
GP88	*S. faecium* LM1	Em^r Rif^r Fus^r	$<3.5 \times 10^{-7}$
GP88	*S. mutans* GS5-Str	Em^r Str^r	$<2.6 \times 10^{-7}$
GP88	*S. pneumoniae* DP1002	Em^r Nov^r	1.2×10^{-6}
S. sanguis			
GP202	*S. pneumoniae* DP1002	Em^r Nov^r	1.2×10^{-6}
GP202	*S. faecalis* JH2-2	Em^r Rif^r Fus^r	$<6.2 \times 10^{-5}$
GP202	*S. pyogenes* D471	Em^r Str^r	$<5.5 \times 10^{-5}$
GP202	*S. faecium* LM1	Em^r Rif^r Fus^r	$<6.2 \times 10^{-5}$
GP202	*S. sanguis* V481	Em^r Rif^r	$<1.6 \times 10^{-6}$

[a] Plating and scoring of transconjugants was done by the agar overlay method (11a, 17); in the overlying layer of the selection plates concentrations were as follows: erythromycin, 2 μg/ml; streptomycin, 500 μg/ml; rifampin, 100 μg/ml; fusidic acid, 100 μg/ml.

[b] CFU of transconjugants divided by CFU of donors.

The flexibility of the system could be increased by trying to improve the conjugation protocol, adapting it to the different streptococcal strains; for instance, *S. sanguis* proved not to be a good donor in filter matings under conditions originally designed for conjugation in *S. pneumoniae* (16). On the other hand, since pDP36 is capable of integrating heterologous DNA within the *tetM* gene, it could be used with virtually any conjugative chromosomal element or conjugative transposon carrying *tetM* (1, 2, 12), thus allowing a different and broader host range of conjugal transfer, than that of Ω6001.

We showed that heterologous DNA can be efficiently integrated into a streptococcal chromosomal element during transformation and then transferred by conjugation in interspecific matings. The Ω6001-pDP36 host-vector system that is described in this work opens new possibilities of gene transfer among streptococci. Recombinant DNA molecules can be subcloned in pDP36 in the region that is heterologous with respect to Ω6001 and integrated into the chromosome of *S. pneumoniae* or *S. sanguis* together with the erythromycin resistance marker. The selection for Em^r Tc^s would allow isolation of clones where the recombinant DNA was stably integrated into the chromosomal element Ω6001. The entire element can then be transferred by conjugation to other streptococcal hosts. This approach has been successfully used to transfer the cloned M6 protein gene to various strains of *S. pyogenes* (Oggioni, Pozzi, and Fischetti, manuscript in preparation).

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