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Genetic and Physiological Analysis of Conjugation in *Streptococcus faecalis*

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In an effort to elucidate the mechanisms of conjugal plasmid transfer in *Streptococcus faecalis*, a genetic analysis of the sex pheromone-dependent tetracycline resistance plasmid pCF-10 was initiated. Rare transconjugants obtained from short matings with wild-type donors not exposed to sex pheromones were screened for increased donor potential in a subsequent mating. From this screening, a mutant plasmid, designated pCF-11, whose transfer functions are expressed in the absence of pheromone induction was isolated. Cells carrying pCF-11 spontaneously clump when grown in broth culture but do not excrete sex pheromones active against wild-type donors. In the course of initial experiments, it was observed that physiological conditions could affect plasmid transfer frequency. Therefore, a set of standardized optimal mating conditions was defined. The experiments carried out to determine these conditions revealed that a transient increase in transfer frequency of about 2 orders of magnitude occurred in early-exponential-phase donor cells. This peak of activity is independent of sex pheromone response, since it was observed with induced or uninduced donor cells carrying either pCF-11 or pCF-10.

In *Streptococcus faecalis*, donor cell aggregation and transfer functions of certain conjugative plasmids are induced by sex pheromones, or clumping-inducing agents (CIA), produced by recipient cells (2–4). Most of the experimental work on sex pheromone-dependent plasmid transfer has been carried out with hemolysin and bacteriocin plasmids (2–4). We recently reported the identification of a CIA-dependent R factor carrying tetracycline resistance (Tet^r) (5). This plasmid, designated pCF-10, is a useful model for a genetic study of conjugation since it carries a selectable marker on a plasmid whose transfer genes can be turned on and off by the addition or removal of CIA. We have begun to isolate a series of mutant derivatives of this plasmid and to analyze the effects of the mutations on the donor phenotype. In the course of these initial genetic studies, occasional variations in plasmid transfer efficiencies which appeared to be related to physiological conditions of the cells in the mating mixtures were observed. In light of these observations, we felt that it would be important to define optimal mating conditions for the demonstration of differences in mating behavior between wild-type and mutant donors. In this paper, we describe the isolation of a mutant plasmid which exhibits a high donor potential in the absence of CIA. We also discuss experiments in which we employed the wild-type and mutant plasmids to determine the effects of growth conditions and cell density on plasmid transfer and CIA response.

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MATERIALS AND METHODS

Bacteria and growth medium. The strains used in this study are listed in Table 1. BYGT medium (5) was employed in all experiments.

Mating experiments. For the initial experiments described in this paper, donor and recipient cells were grown to late exponential phase in BYGT medium. A 0.5-ml amount of the recipient culture was mixed with 0.05 ml of donor cells and 4.5 ml of fresh BYGT medium. After appropriate incubation at 37°C, the mixtures were plated on BYGT agar with antibiotics selective for donors, recipients, or transconjugants. CIA induction of donor cells was carried out as previously described (5). Antibiotics used in selective plates (in micrograms per milliliter) and their sources were as follows: rifampin, 50; tetracycline, 8; streptomycin, 500 (all from Sigma); fusidic acid, a gift from W. O. Godtfredsen, Leo Pharmaceutical, 50; and spectinomycin, a gift from J. Grady, The Upjohn Co., 250. Modifications of the basic mating protocol are described below.

CIA assays. CIA assays were carried out by a microtiter method as described previously (5).

RESULTS AND DISCUSSION

When a donor strain carrying wild-type pCF-10 (and not exposed to CIA) was mated with

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TABLE 1. Strains used in this study

Derivation and strain	Chromosomal markers[a]	Plasmids	Comments (reference)
SF-7			
SF-7		pCF-10, pCF-20	Clinical isolate (5)
SF-7-S	Str^r	pCF-10, pCF-20	Spontaneous mutant of SF-7 (5)
7C-5	Str^r	pCF-20	SF-7-S cured of pCF-10 (5)
7C-5R	Str^r Rif^r	pCF-20	Spontaneous mutant of 7C-5
7C-5R11	Str^r Rif^r	pCF-11, pCF-20	This paper
JH2			
JH2-2	Rif^r Fus^r		(7)
JH2-SSp	Str^r Spc^r		(3)
JH2-2P10	Rif^r Fus^r	pCF-10	(5)
JH2-2P11	Rif^r Fus^r	pCF-11	This paper
OG1			
OG1-SSp	Str^r Spc^r		(3, 6)
OG1-SSpP10	Str^r Spc^r	pCF-10	This paper
OG1-SSpP11	Str^r Spc^r	pCF-11	This paper

[a] Abbreviations: Str^r, streptomycin resistance; Spc^r, spectinomycin resistance; Rif^r, rifampin resistance; Fus^r, fusidic acid resistance.

recipients for 10 to 15 min as described in Materials and Methods, the frequency of transfer was generally less than 10^{-6}. Plating 0.1 ml of such mixtures on plates selective for Tet^r transconjugants yielded less than 10 colonies per plate. When 50 of the rare transconjugants obtained from a 10-min mating of SF-7 with 7C-5R were tested for the ability to transfer Tet^r in a second round of mating, one isolate showed an increase in donor potential over the wild type. This mutant plasmid, designated pCF-11, transferred at frequencies of 10^{-4} to 10^{-5} per donor in 15-min matings, whereas transfer of pCF-10 was virtually undetectable before 60 min of mating time. After 60 min, the differences in transfer frequency were somewhat less, presumably because the wild-type cells could respond to CIA produced in the mating mixture. We concluded that the genetic alteration in the mutant is located on the plasmid because the derepressed transfer phenotype was always transferred along with pCF-11. Strains carrying this plasmid spontaneously clump in liquid culture. Thus, it would appear that the aggregation substance (4) responsible for clumping is synthesized constitutively by cells carrying this plasmid. Agarose gel electrophoresis of plasmid preparations from pCF-11-carrying cells did not reveal any gross differences in size or copy number between pCF-11 and pCF-10 (data not shown).

According to the published model for the genetics of CIA response (4), the phenotype of pCF-11 could result from either of two alterations in the plasmid. The aggregation genes of the plasmid could be fully expressed in the absence of exogenous CIA, or the gene(s) responsible for blockage of CIA production by plasmid-carrying cells could be defective. In the case of the second possibility, pCF-11-carrying cells would excrete CIA and thereby stimulate themselves to clump. When culture filtrates of cells carrying pCF-11 were tested for CIA activity against responder cells carrying the wild-type pCF-10, no activity was detected. Therefore, it appears that the mutant plasmid expresses its aggregation genes without stimulation by CIA.

We have previously demonstrated that the expression of the fertility functions of pCF-10 are affected by the genetic background of the host cell (5). The effects of host background on expression of fertility functions of the mutant and wild-type plasmids have been compared (Table 2). Consistent with our previous observations (5), pCF-10 transferred efficiently from an SF-7 or OG1 host cell and also determined a clumping response to CIA. In a JH2-2 host, responses to CIA and plasmid transfer were both very poor. In contrast, pCF-11 transferred with a high efficiency and exhibited its self-clumping phenotype in all three genetic backgrounds. These results indicate that the conjugal transfer functions of pCF-10 could be expressed in JH2-2 if an exogenous CIA signal was not required. In designing experimental studies of the CIA signal reception mechanism, it may be worthwhile to consider the apparent differences between JH2-2 and other *S. faecalis* strains.

TABLE 2. Expression of pCF-10 and pCF-11 fertility functions in various *S. faecalis* hosts

Strain	Host, plasmid	Frequency of transfer in 2-h broth mating	Clumping response to CIA[a]
SF-7	SF-7, pCF-10	2×10^{-4}	+
7C-5R11	SF-7, pCF-11	8×10^{-4}	NT[b]
JH2-2P10	JH2-2, pCF-10	7×10^{-6}	−
JH2-2P11	JH2-2, pCF-11	6×10^{-3}	NT
OG1-SSpP10	OG1-SSp, pCF-10	3×10^{-4}	+
OG1-SSpP11	OG1-SSp, pCF-11	5×10^{-3}	NT

[a] As determined in a microtiter clumping assay (5).
[b] NT, Not testable because the strain clumped in the absence of CIA.

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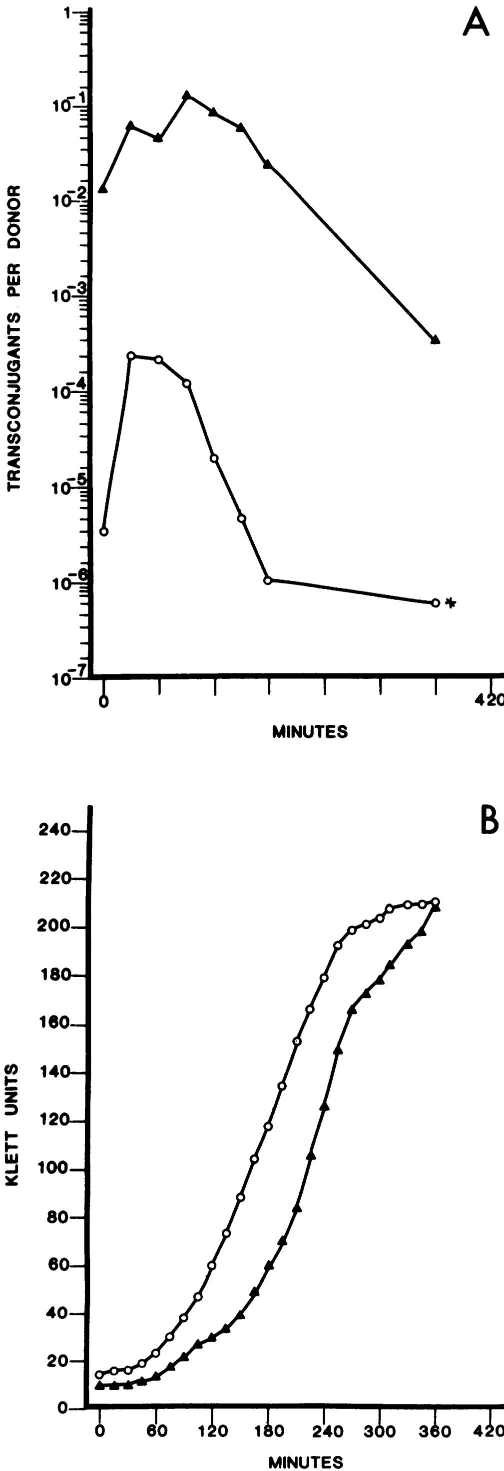

FIG. 1. Transfer of pCF-10 (○) and pCF-11 (▲) at various phases of growth. (A) Frequency of transfer of Tet^r in a 15-min mating from OG1-SSpP10 or OG1-SSpP11 donor cells removed from the culture at various times after a 25-fold dilution of an overnight culture into fresh medium. The cell concentrations in all matings were adjusted to approximately 6×10^6 donors per ml and 6×10^7 JH2-2 recipients per ml. *, No transconjugants detected; frequency, $<7 \times 10^{-7}$. (B) Growth curve of the donor cultures as determined in a Klett-Summerson colorimeter with a no. 54 green filter.

Several preliminary experiments in which the transfer frequencies of pCF-10 and pCF-11 were compared under various conditions suggested that the efficiency of plasmid transfer might be affected by factors independent of CIA response, such as growth phase or the concentrations of the cells participating in the mating. To define a standard set of optimal mating conditions that would allow clear distinction between wild-type and mutant mating behavior, we first tested the ability of OG1-SSp donor cells carrying either pCF-10 or pCF-11 to transfer Tet^r in various phases of growth. In all matings, exponential-phase JH2-2 cells were employed as recipients. Overnight cultures of donor cells were diluted to about 10^7 colony-forming units per ml, and samples were removed at early, middle, and late exponential phase and after reaching stationary phase. They were immediately tested for donor ability in a 15-min mating.

Figure 1 shows that, as expected, cells carrying pCF-11 were much more effective donors in short matings with no preinduction by CIA. However, in early exponential phase, both strains transferred their plasmids at frequencies of 2 to 3 orders of magnitude higher than in stationary phase. The peak of pCF-10 donor potential was somewhat sharper than that observed with the pCF-11 donors. As shown in Fig. 1, cells carrying pCF-10 initiated exponential growth and also reached stationary phase somewhat more quickly than did isogenic cells carrying pCF-11. In both cases, the peak of donor ability appeared to coincide with the beginning of exponential growth. Since transfer of both the wild-type and mutant plasmids showed a similar dependence on growth phase, the effects observed must have been independent of the genetic differences between these plasmids. In carrying out a number of repetitions and variations of this type of experiment, we found that donor cells at this peak phase of growth invariably showed a 50- to 150-fold increase in transfer efficiency relative to that of stationary-phase cells. We also tested the effects of varying the growth phase of the recipient cells. No significant differences in transfer efficiency attributable to the growth phase of the recipients were found.

The peaks in transfer frequency observed with the early-exponential-phase *S. faecalis* donor

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cells resembled streptococcal competence peaks (see references 8 and 9 for reviews), which result from the production of soluble-competence-inducing factors that can be isolated from culture filtrates of cells competent for transformation. We attempted to increase the frequency of transfer of stationary-phase donor cells by adding culture filtrates from early-exponential-phase donors to mating mixtures containing the late donor cells. We also attempted to increase the donor potential of late cells carrying pCF-11 by adding early cells carrying pCF-10. (This experiment was feasible because pCF-11 transferred much more frequently than pCF-10, and the transconjugants could be tested for the self-clumping phenotype to verify that they actually acquired pCF-11.) No stimulation of transferability of the late donor cells was observed in any of these experiments, arguing against the involvement of soluble mating factors in this phenomenon.

The ability of wild-type and mutant donors in different growth phases to exhibit increased plasmid transfer in response to CIA was measured. As shown in Table 3, wild-type donors showed higher frequencies of transfer in 10-min matings and better CIA induction when overnight cultures were used directly than when the cells were grown for 80 min before induction, indicating that the total incubation time of donor cells (after dilution of a stationary culture into fresh medium) is a critical determinant in the mating potential of that donor culture. Cells incubated for 80 min before the 60-min CIA induction apparently grew past the peak of donor potential (although they still showed a 60-fold increase in transfer frequency over cells from a culture of the same age not exposed to CIA). In the case of pCF-11, the transfer frequencies were very high under all conditions, consistent with the broad peak of donor ability shown in Fig. 1. It is noteworthy that transfer frequencies of 1×10^{-1} to 5×10^{-1} in 10-min matings were observed with pCF-11 under these conditions. A number of prospective biochemical and microscopic experiments designed to further elucidate the detailed mechanisms of plasmid transfer would require a significant proportion of the donor cells to be actively participating in conjugation during experimental measurements. pCF-11 would therefore appear to be a good candidate for such experimentation. The fact that transfer frequencies of >1 were observed in 2-h matings indicates that multiple rounds of transfer occurred. Thus, it should be feasible to determine whether streptococcal conjugation is culminated by an active disaggregation process, which is known to occur in gram-negative systems (see reference 1 for review).

We subsequently tested the effects of the concentration of cells in the mating mixtures on plasmid transfer efficiency. The frequency of transfer did tend to increase with cell concentration (Table 4); however, at concentrations of 10^8 donors per ml and 10^9 recipients per ml, there was a reduced difference between CIA-induced and uninduced donors. Therefore, the use of early-exponential-phase donors, along with maintaining concentrations of about 10^7 donors per ml and 10^8 recipients per ml in mating mixtures, would appear to give a high frequency

TABLE 4. Effects of cell concentrations on plasmid transfer[a]

Approximate cell concn (no./ml)		Transfer of pCF-10 donors in a 10-min mating	
Donors	Recipients	Uninduced	CIA induced
10^6	10^7	5.6×10^{-6}	1.1×10^{-3}
10^7	10^8	1.5×10^{-6}	1.6×10^{-2}
10^8	10^9	4.3×10^{-5}	1.6×10^{-2}

[a] OG1-SSpP10 donor cells were diluted from an overnight culture to 10^7 colony-forming units per ml and grown for 80 min in BYGT medium. JH2-2 recipient cells were grown to mid-exponential phase. Both cultures were concentrated by centrifugation, diluted to desired concentrations, and incubated at 37°C during CIA induction of donor cells. After a 1-h induction, 10-min matings were carried out.

TABLE 3. Effects of CIA induction on transfer of pCF-10 at various phases of growth[a]

Donor strain	Time (min) of donor incubation before induction	No. of transconjugants per donor with indicated time (min) of mating			
		CIA induced		Uninduced	
		10	120	10	120
OG1SSp P10	0	3.2×10^{-2}	3.9×10^{-1}	3.9×10^{-4}	4.1×10^{-1}
OG1SSp P10	80	7.2×10^{-4}	9.9×10^{-1}	1.2×10^{-5}	5.7×10^{-1}
OG1SSp P11	0	3.0×10^{-1}	1.4	1.9×10^{-1}	8.5×10^{-1}
OG1SSp P11	80	4.1×10^{-1}	4.6×10^{-1}	3.8×10^{-1}	1.46

[a] Overnight cultures of donor cells were diluted into fresh BYGT medium and grown for the times indicated before a 60-min CIA induction as previously described (5). Donor and recipient concentrations in mating mixtures were approximately 5×10^6 and 1×10^8 colony-forming units per ml, respectively.

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of transfer and demonstrate the maximum difference between induced and uninduced donor cells.

Taken together, the results presented here enabled us to define a set of standard mating conditions which should be useful in analysis of various mutants altered in CIA response and plasmid transfer phenotypes. This work also reveals that the physiological state of *S. faecalis* cells, as determined by their growth phase, has important effects on their ability to act as donors of plasmid DNA, distinct from those induced by sex pheromones.

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