Interspecies Bacterial Conjugation by Plasmids from Marine Environments Visualized by *gfp* Expression

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Horizontal transmission of DNA between different species may have played an important role in evolutionary history. Gene transfer encoded by bacterial plasmids has occurred between distantly related bacterial species; it may have occurred between species of different kingdoms. We have developed a system to detect conjugal plasmid transfer in situ based on the expression of the green fluorescent protein (GFP). Plasmids were tagged with the *gfp* gene under the control of a *lac* promoter. These plasmids were placed in a *Pseudomonas putida* strain carrying a chromosomal *lac* repressor. In conjugation mixtures, the donor strain remained nonfluorescent, but any newly formed transconjugant cell fluoresced. Unlike other assays for conjugation, this assay is sensitive enough to detect the formation of a single transconjugant a short time after it occurs. We tested for the transfer of three plasmids that were originally exogenously isolated from marine bacterial communities. Eleven of the 19 different eubacterial recipients formed transconjugants, including a species only distantly related to the donor, *Planctomyces maris*. The results imply that interspecies gene transfer mediated by conjugation is common in natural environments, and may explain why similar DNA sequences can be found among distantly related bacterial species.

Introduction

Horizontal gene exchange is of importance in the adaptation and evolution of bacteria (reviewed in Syvanen 1994). When bacterial DNA sequences and their GC contents and/or codon usage have been analyzed, many cases of possible horizontal gene transfer events have been revealed. For example, as much as 600 kb of the *Escherichia coli* genome could have been acquired by horizontal transfer (Lawrence and Ochman 1997). Many bacterial genes appear to have had "foreign" ancestry: the *E. coli phoN* gene (Groisman, Saier, and Ochman 1992), the *E. coli cob* operon (Lawrence and Roth 1995), the *Salmonella typhimurum* sialidase gene (Hoyer et al. 1992), the *Erwinia chrysanthemi celY* gene (Guiseppi et al. 1991), the *Brevibacterium* sp. *amiE* gene (Soubrier et al. 1992), and, finally, the *Frankia* spp. *nifK* gene (Hirsch et al. 1995). In addition, examples of genes horizontally transferred from prokaryotes to eukaryotes, and vice versa, have been suggested (Smith, Feng, and Doolittle 1992; Kidwell 1993). The dissemination of antibiotic and other resistance genes among bacteria also shows the impact of horizontal gene transfer in the adaptation and evolution of bacteria (see, for example, Levy and Miller 1989).

Bacterial conjugation is one gene transfer mechanism that is important in the horizontal flow of genetic information (reviewed in Mazodier and Davies 1991). Plasmids have been identified in a wide range of bacterial species, and transfer between distantly related bacteria has been demonstrated. Examples of conjugative plasmids involved in transkingdom DNA transfer between bacteria and yeast (Heinemann and Sprague 1989) or plants (reviewed in Farrand 1993) are also known.

Key words: plasmids, horizontal transfer, broad host range, interspecies, marine, *gfp*.

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The majority of the plasmids examined so far are of clinical importance, and data on plasmids from natural environments are relatively scarce. Further work is needed to explore the impact plasmids have on bacterial species as "importers of foreign DNA" in clinical and natural environments.

Traditionally, plasmid transfer is detected by growth of transconjugants on selective media, although molecular methods also are available. Selective growth of transconjugants requires that the plasmids are stable for several generations in the recipient species. This is a drawback, since recombination events between the plasmid and the host genome may occur even if the plasmid is not stably maintained. New methods for detection of gene transfer have recently been designed based on the expression of *lacZ* (Jaenecke et al. 1996) and *gfp* (Christensen, Sternberg, and Molin 1996). The latter system takes advantage of the strong fluorescence from the green fluorescent protein (GFP), originally isolated from *Aequorea victoria* (Chalfie et al. 1994). GFP has the advantage that there is no need for an additional substrate for its expression, and it can be expressed in both eukaryotic and prokaryotic cells. We have developed a GFP-based system to detect conjugal plasmid transfer that differs from that previously published (Christensen, Sternberg, and Molin 1996) in the respect that no special features are needed for *gfp* expression in the recipient bacterium.

The question addressed in this study is to what extent naturally occurring plasmids have the potential for interspecies transfer. We examined conjugative plasmids previously isolated from marine bacterial communities. Nineteen different eubacterial species (10 marine isolates) from six different major phylogenetic lineages were tested as recipients for three different plasmids.

Material and Methods
Bacterial Strains and Culture Media

The bacterial strains used and their phylogenetic positions are listed in table 1. *Pseudomonas putida*

Table 1
Bacterial Strains

Phyla (subdivisions) and Strains	Characteristics[a]	Growth Conditions[b]	Source/Reference[c]
Proteobacteria (gamma group)			
Pseudomonas putida KT2442	*lac I*q, *hsd*R, Rifr	LB15/25°C	S. Molin
Pseudomonas putida KT2440	*hsd*R, Nxr	LB15/25°C	S. Molin
Aeromonas hydrophila		NA/30°C	CCUG 14551
Alteromonas atlantica................	Marine isolate	BMM/20°C	NCIMB 301
Escherichia coli LE392	Rifr	LB/37°C	Bale, Fry, and Day (1988)
Listonella anguillarum	Marine isolate	SWA/20°C	NCIMB 2129
Pseudomonas fluorescens.............		NA/20°C	CCUG 1253
Serratia marcescens		NA/37°C	CCUG 1647
Vibrio fischerii.....................	Marine isolate	SWYP/20°C	L. Holmquist
Vibrio sp. S14	Marine isolate	BMM/25°C	S. Kjelleberg
Proteobacteria (alpha group)			
Caulobacter maris..................	Marine isolate	BMM/20°C	NCIMB 2209
Hyphomonas neptunicum	Marine isolate	BMM/25°C	NCIMB 2023
Gram-positive bacteria (high GC)			
Corynebacterium sp................	Marine isolate	NA/20°C	NCIMB 2025
Micrococcus luteus		NA/30°C	CCUG 5838
Gram-positive bacteria (low GC)			
Bacillus subtilis.....................		NA/30°C	CCUG 16313
Listeria murrayi		NA/30°C	CCUG 4984
Planococcus sp...................	Marine isolate	BMM/20°C	NCIMB 1097
Bacteroides, Flavobacteria			
Flavobacterium brevi		NA/25°C	CCUG 7320
Planctomyces and relatives			
Planctomyces maris	Marine isolate	BMM/25°C	NCIMB 2232

[a] Rif, rifampicin; Nx, nalidixic acid.

[b] LB, Luria-Bertani medium; LB15, LB supplemented with NaCl to a final concentration of 1.5%; NA, nutrient agar; BMM, bacto marine medium 2216; SWA, sea water agar; SWYP, sea water yeast peptone medium.

[c] CCUG, Culture Collection of University of Göteborg; ATCC, American Type Culture Collection; NCIMB, National Collection of Industrial and Marine Bacteria.

KT2442 *lac I*q was used as the donor strain for all plasmids in the conjugation experiments. Nineteen different bacterial species were tested as recipients, of which 10 were marine isolates (table 1). The culture media used were Luria-Bertani medium (LB) (Maniatis, Fritsch, and Sambrook 1982), LB supplemented with NaCl to a final concentration of 1.5% (LB15), nutrient agar (NA) (Oxoid CM3), bacto marine medium 2216 (BMM) (Difco), sea water agar (SWA), and sea water yeast peptone medium (SWYP), the last two described in the National Collection of Industrial and Marine Bacteria (NCIMB) catalog of strains. The media used for different strains are listed in table 1. All strains were grown overnight, except *Hyphomonas neptunicum* and *Planctomyces maris,* which were grown for 2 and 10–14 days, respectively. All cultures were grown with shaking at the temperature listed in table 1. The media for the agar plates used in the conjugation experiments were 10-fold diluted; 100 ml of respective liquid media was added to either 900 ml 0.5% NaCl autoclaved with agar for NA media or 900 ml of aged sea water autoclaved with agar for the other three media. Growth media was supplemented with streptomycin (500 μg/ml), nalidixic acid (200 μg/ml), kanamycin (25 μg/ml), and/or rifampicin (100 μg/ml) when necessary.

gfp-Tagged Plasmids

The plasmids examined in this study were originally obtained by exogenous isolation (Fry and Day 1990) from marine bacterial communities in the model recipient *P. putida* UWC1 with selection for mercury resistance. Plasmids pB7 and pB9 (structural group 1) differ slightly in their RFLP patterns, while pBF1 (structural group 8) has a different structure (Dahlberg et al. 1997).

The construction of the expression system and the insertion of *gfp* into the plasmids are described elsewhere (Andreasen 1997). Briefly, a construct was made where the wild-type *gfp* gene is cloned downstream of the *lacP*A1-04/03 promoter. The *lacP*A1-04/03::*gfp* was then inserted into a pUT Tn*5*-based insertion delivery plasmid (Herrero, de Lorenzo, and Timmis 1990). The pUT plasmid was subsequently transferred to *P. putida* containing pB9, pBF1, or pB7, and transposition of the *gfp* hybrid transposon from pUT to these plasmids occurred. pB9, pBF1, and pB7 carrying the *lacP*A1-04/03::*gfp* cassette were selected and further conjugated into a *P. putida* KT2442 strain. Since this KT2442 strain carries a chromosomal *lac I*q repressor, *gfp* is not expressed in this strain. When the plasmid is transferred to other bacteria, this repression is released, which makes it possible to detect the fluorescent GFP protein in the transconjugant cells. The tagged plasmids also code for kanamycin resistance, since the *npt* gene is a part of the hybrid mini-Tn*5*.

Conjugation Experiment

Donor and recipient cells from liquid cultures were mixed by quick vortexing in ratios of 1:100. Drops of 200 μl from the mixtures were spread on sterile polycarbonate membranes. Filters were placed on 10-fold-diluted solid media suitable for the recipient cells (table

Table 2
Frequency of Plasmid Transfer[a] to Different Recipient Species from the Donor Strain, *Pseudomonas putida* KT2442, Based on Detection of GFP Fluorescence

	PLASMID		
RECIPIENT STRAIN	pB7	pBF1	pB9[b]
Proteobacteria (gamma group)			
Pseudomonas putida	2×10^{-3}	5×10^{-1}	+
Aeromonas hydrophila	$<1 \times 10^{-4}$	3×10^{-2}	+
Alteromonas atlantica	—	—	
Deleya marina	8×10^{-4}	7	+
Escherichia coli	5×10^{-4} [c]	1×10^{-2} [c]	+
Listonella anguillarum	—	—	
Pseudomonas fluorescens	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$	
Serratia marcescens	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$	
Vibrio fischerii	1×10^{-3}	4×10^{-3}	+
Vibrio sp. S14	6×10^{-4}	2×10^{-2}	+
Proteobacteria (alpha group)			
Caulobacter maris	$<1 \times 10^{-4}$	2×10^{-3}	
Hyphomonas neptunicum	—	5×10^{-3}	
Gram-positive bacteria (high GC)			
Corynebacterium sp.	—	—	
Micrococcus luteus	—	—	
Gram-positive bacteria (low GC)			
Bacillus subtilis	—	—	
Listeria murrayi	—	—	
Planococcus sp.	—	—	
Bacteroides, Flavobacteria			
Flavobacterium brevi	—	—	
Planctomyces and relatives			
Planctomyces maris	—	$<1 \times 10^{-4}$ [d]	

[a] The frequency represents the number of transconjugants (determined by their fluorescence) after 24 h of incubation, divided by the number of donor cells initially added. Average of two independent experiments.

[b] Transfer is determined when number of fluorescent cells greatly exceeds the fluorescence observed on the control membranes.

[c] Transconjugants were enumerated by selective plating.

[d] Fluorescence from recipient cells was detected after 4 days of incubation.

1). Control membranes were made in the same manner for all strains separately, with the same amount of bacteria on the type of plate used for each conjugation pair. The conjugation membranes were incubated at room temperature for up to 7 days. Each time series was repeated two to four times starting from new cultures. The conjugation pairs showing fluorescent transconjugants at a higher frequency than 1×10^{-4} (table 2) after 1 day of incubation were resuspended in the last two time series after 1 day and filtered onto a new membrane for exact enumeration of fluorescent cells.

Membranes were transferred to microscopic slides, and fluorescent cells were detected in an epifluorescence microscope (Olympus) using blue excitation light and a $100\times$ objective. A minimum of 20 view fields were examined on each membrane. The daily examination of membranes was carried out for up to 3 days after the first fluorescent cell appeared. Mixtures for which no fluorescence could be detected were examined until day 7. The three plasmids were also transferred to *P. putida* KT2440, which served as the donor strain in conjugation experiments to *E. coli,* and, in this case, the transconjugants were recovered on selective media.

The number of transconjugants that could be detected at the single-cell level by their fluorescence was compared to the number found by selective plating on kanamycin (to select for the plasmid) and streptomycin amended media. This comparison was made for two recipient species, *Vibrio* sp. S14 and *Deleya marina* (streptomycin spontaneous mutants).

Results

Fluorescent transconjugants appeared after 24 h of incubation in mixtures with several of the recipients (table 2). The only case in which fluorescent cells appeared later than 24 h was that of *P. maris,* which required 4 days of incubation.

Transfer of plasmid pBF1 was detected to 11 of the 19 tested recipient strains, including species from the proteobacteria gamma and alpha groups and the distantly related *Planctomyces* species (table 2). Figure 1 shows fluorescent transconjugants formed with three different recipient species. The highest transfer frequency was obtained with *D. marina* and was 10-fold higher than in the intraspecies conjugation to *P. putida* KT2440 (table 2).

Plasmid pB7 transferred to nine of the recipient species. Those recipients that received pB7 were also able to receive pBF1. Transfer of pB7 could not be detected to *H. neptunicum* or *P. maris,* in contrast to pBF1. The transfer frequencies of pB7 were generally much lower than those of pBF1. The highest frequencies to other species were obtained with *V. fisherii, Vibrio* sp. S14, and *D. marina* as recipients (table 2).
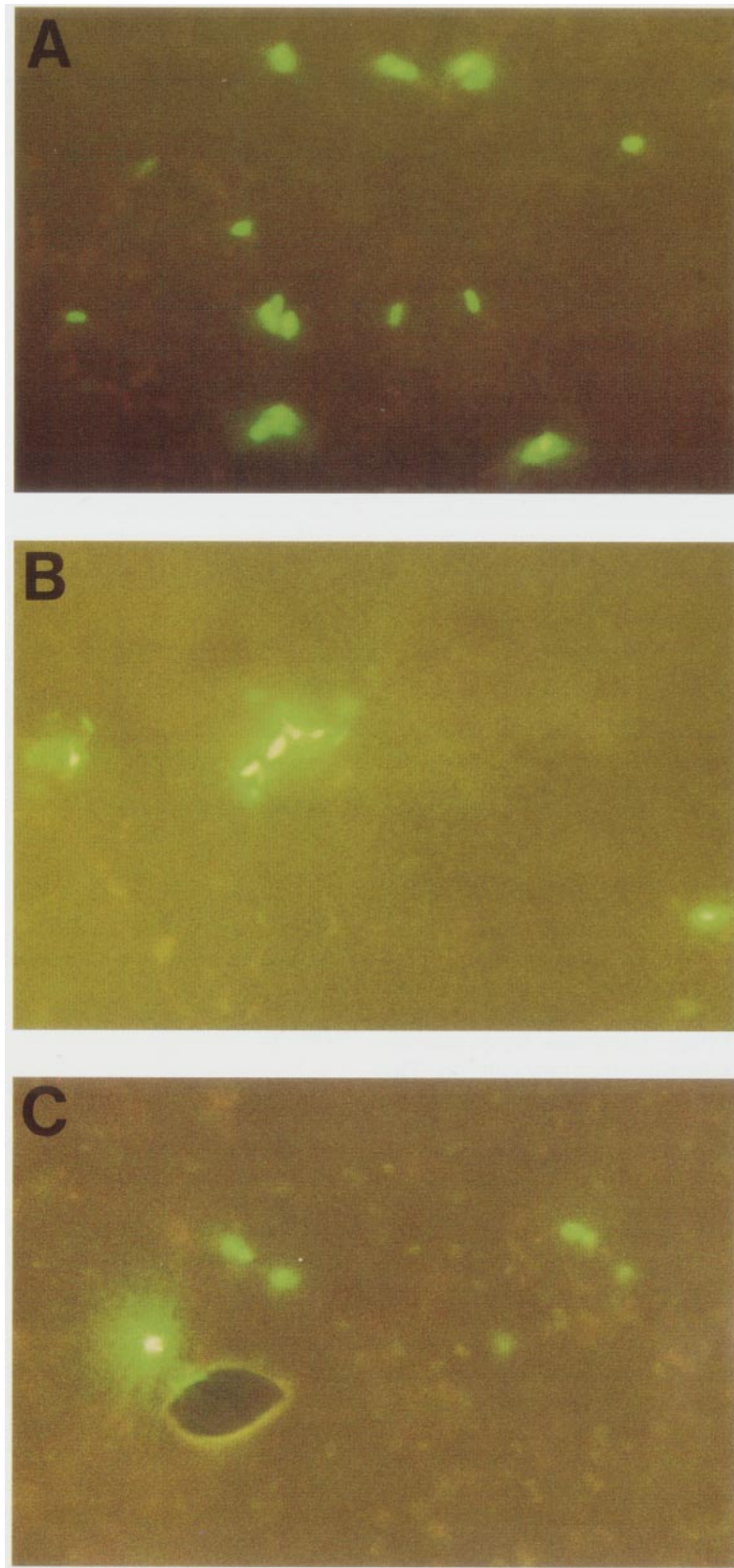
FIG. 1.—Photographs show fluorescence from the *gfp*-tagged plasmid pBF1 in the recipient species (*A*) *Vibrio* sp. S14, (*B*) *Hyphomonas neptunicum*; and (*C*) *Planctomyces maris* in conjugation mixtures with the donor strain *Pseudomonas putida.*

Transfer of plasmid pB9 was more difficult to assess due to background fluorescence from the donor strain that became stronger for each day of incubation. Transfer could be determined to six of the species in the proteobacteria gamma group, to which plasmid pB9 transferred at relatively high frequencies (table 2). In the other recipient species, it was not possible to distinguish transconjugants.

Incubations of the donor strain carrying either pBF1 or pB7 in the absence of recipients did not give rise to any fluorescent cells on any of the media used. Control membranes with the different recipient species showed no fluorescence at any time.

The fluorescence was strong from the transconjugants formed with all species except *P. fluorescens*. The fluorescence from these transconjugants did not increase with time but, rather, faded after 5 days of incubation.

The transfer frequency values were higher when the transconjugants were recovered on selective media than they were with direct detection of GFP fluorescence. In *Vibrio* sp. S14, 1%–5% of the number of transconjugants recovered on selective media was detected by direct microscopy after 24 h for both plasmid pBF1 and plasmid pB7. The same was true for the pBF1 transconjugants of *D. marina,* whereas 15% of the pB7 transconjugants recovered on selective media were detected in situ. The difference between direct detection and selective growth, however, decreased after prolonged incubation.

Discussion

In this study, we show that high levels of promiscuity are found among plasmids from a collection of conjugative plasmids previously isolated by exogenous isolation from marine bacterial communities (Dahlberg et al. 1997). The results suggest that interspecies barriers for gene exchange can be low in complex bacterial communities, as these plasmids are transferred to a wide range of different species.

The plasmid constructs, with *gfp* expressed from a *lac* promoter, works efficiently in many bacterial species. An analog to this system was previously described in which the Pr promoter-operator from bacteriophage lambda was used to control the expression of a *lacZ* gene inserted on conjugative plasmids (Jaenecke et al. 1996). The expression of the *lacZ* construct was shut down by two chromosomally located repressors in the donor strain. One advantage with *gfp* is that there is no need for additional substrates for its expression. The GFP fluorescence is also strong, which makes it possible to study conjugation in microbial systems on a single-cell level and in situ.

Transfer of the plasmids was primarily detected to species within the proteobacterial phylum but also to those of the phylum containing planctomycetes. No transfer was detected to gram-positive bacteria or flavobacteria. The plasmids pB7 and pBF1 were both transferred to 9 of the 12 species from the proteobacteria apha and gamma groups (table 2). In addition, plasmid pBF1 formed transconjugants with *H. neptunicum* and

with *P. maris* (table 2). The planctomycetes (for a review, see Fuerst 1995) have characteristics that are not present or very uncommon in other eubacteria, including, for example, a budding mode of division, a membrane-bounded nuclear body, and lack of a cell wall containing peptidoglycan. Two different theories of the phylogenetic position of this group exist based on 16S rRNA analysis, one implying that the planctomycetes represent an ancient group of bacteria descending from a group branching close after that the eubacteria diverged from the progenote, and the other suggesting that they represent a very rapidly evolving group of bacteria (Fuerst 1995). The transfer potential of plasmid pB9 was harder to determine due to its leaky behavior in the donor strain. Transfer was documented to six of the species in the proteobacteria gamma group (table 2).

We obtained high transfer frequencies to many different species; up to seven transconjugants per donor cell initially added were detected. It should, on the one hand, be kept in mind that the values include growth of the cells and overestimate the transfer rate. On the other hand, after 24 h, it is possible to microscopically detect 1%–15% of the number of transconjugants by their fluorescence, compared to the number of transconjugants recovered on selective plates. A certain amount of GFP must be expressed in the cell to make it detectable, and GFP detection is also delayed for approximately 4 h due to an oxidative step required to make GFP fluorescent (Heim, Prasher, and Tsien 1994). Comparisons of exact transfer frequencies between different plasmids and/or species are therefore difficult to make. All transconjugant colonies recovered on selective media were fluorescent. The difference in numbers of transconjugants detected on selective media and by fluorescence may be explained by the delay in development of GFP fluorescence. No transfer was detected to eight of the recipient species. Obvious reasons for the absence of GFP fluorescence in these species are that the plasmids were not transferred or that the *lac* promoter does not function. It is known that genes from gram-negative bacteria are expressed at very low levels, if at all, in gram-positive strains. An extended incubation time might serve to overcome this deficiency by accumulation of GFP over time.

Plasmids from the broad-host-range IncP incompatibility group have previously been demonstrated to efficiently transfer to a wide range of gram-negative hosts using other methods (Guiney and Lanka 1989). The genes that constitute the transfer system of IncP plasmids have a wider host range that also includes gram-positive bacteria, *Mycobacteria, Bacteroides,* and cyanobacteria (for an overview see Guiney 1993). Other well-characterized broad-host-range plasmids such as IncN and IncW can transfer into a number of gram-negative bacteria (Iyer 1989; Valentine and Kado 1989). The three plasmids used in this study do not correspond to any of 14 probes from of a collection of probes previously designed to characterize incompatibility groups (Couturier et al. 1988), including the broad-host-range plasmids P, W, N, and Q (Dahlberg et al. 1997).

Our results do not necessarily mean that plasmids that have entered new host species can be maintained for many generations. The transfer systems of conjugative plasmids, for example, often allow a wider host range than do the replication systems (Guiney 1993). The important conclusion from an evolutionary point of view is that opportunities for recombination with genes from other species are present and are not rare.

Acknowledgments

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