INTRODUCTION

Horizontal gene transfer and recombination are important processes in the adaptive evolution of prokaryotes (Arber, 2000; Ochman et al., 2000). These processes can lead to the formation of new alleles as well as to the integration of genetic information evolved in other organisms. Horizontal transfer can encompass chromosomal DNA and plasmids, and will promote exchange within and between species. Among the major transfer mechanisms, conjugation and transduction are processes directed by genetic elements like plasmids, transposons or viruses often considered as infectious or parasitic elements (Levin & Bergstrom, 2000). In contrast, the active uptake of DNA present in the environment by cells during natural transformation is performed by the coordinated functions of a large group of genes distributed over the cellular genome. Transformation may therefore be considered as the genuine prokaryotic gene transfer mechanism (Lorenz & Wackernagel, 1994; Dubnau, 1999). Several barriers to interspecific horizontal transfer of chromosomal DNA have been identified. A decline of the nucleotide sequence identity between donor and recipient leads to a decrease of the integration of donor DNA by homologous recombination in a log-linear relation.
double-stranded DNA (Redaschi & Bickle, 1996). In fact, in the three naturally transformable bacteria studied in this respect (Bacillus subtilis, H. influenzae and Streptococcus pneumoniae), a strong effect of restriction was not seen (Bron et al., 1980; Stuy, 1976; Lacks & Springhorn, 1984). Here we examined the influence of the source of DNA on the natural transformation of Pseudomonas stutzeri JM300. Members of the species P. stutzeri have been found worldwide in terrestrial and aquatic habitats, and a large fraction of the isolates obtained from a variety of environmental samples are naturally transformable (Sikorski et al., 1999, 2002h). Using cloned P. stutzeri DNA for transformation we found that DNA replicated in Escherichia coli was much less effective in natural transformation and electroporation in strain JM300, while transformation of restriction-deficient mutants derived from JM300 was independent of the source of DNA.

**METHODS**

**Strains, plasmids and media.** The strains and plasmids used in this work are listed in Table 1. Strains were grown in LB medium (Sambrook et al., 1989) at 37°C. The minimal medium was a succinate medium (MS) as described (Lorenz & Wackernagel, 1991). Antibiotics were added to media if required: gentamicin (Gm; 5 μg ml⁻¹ in liquid media, 15 μg ml⁻¹ in agar medium), tetracycline (Tc; 10 μg ml⁻¹), chloramphenicol (Cm; 20 μg ml⁻¹), kanamycin (Km; 60 μg ml⁻¹), ampicillin (Ap; 1000 μg ml⁻¹). Strain JB12 was obtained from JM300 by allelic exchange of the chromosomal JM300 DNA (Sikorski et al., 1998). Strain CB61 was obtained by natural transformation of strain CB6 with chromosomal DNA of JB12. The plasmid pUCP7-4 was pUCP19 (Schweizer, 1991) with the tetA tetR genes from Tn10 cloned into the Scul site of the bla gene. The plasmid also contained, in its multiple cloning site, a 4-7 kb EcoRI-BamHI fragment from pEB1 (Brunschwig & Darzins, 1992) covering the T7 DNA polymerase gene under the control of the lacUV5 promoter and the lacI gene with the lacI promoter. E. coli SF8 was used to prepare DNA of various plasmids which were introduced by electroporation as described by Ostendorf et al. (1999).

**Treatment of cells with N-methyl-N'-nitro-N-nitosoguanidine.** Exponential-phase cells were treated with 75 μg N-methyl-N'-nitro-N-nitosoguanidine ml⁻¹ for 10 min at 37°C as described by Ostendorf et al. (1999). The cell survival was about 20%. About four generations were allowed for segregation.

**Natural transformation assay.** The plate transformation procedure (Meier et al., 2002) was used. The plates with cells and DNA were incubated for 20 h at 37°C. Transformation frequencies are expressed as transformed cells per total viable count.

**DNA manipulations.** Chromosomal DNA from P. stutzeri was isolated using the Qiagen Genomic-tip 100G. For preparation of plasmid DNA from E. coli or P. stutzeri the Qiagen Plasmid Kit was employed. DNA restriction and cloning of DNA fragments followed standard procedures. The fd phage was isolated from R408 helper phage-infected E. coli KK2186 pPM1 cells and purified by ultracentrifugation and treatment for 15 min with DNase I as described (Meier et al., 2002). The DNA from fd was purified by extraction with phenol four times (Sambrook et al., 1989). DNA fragments were recovered from agarose gels by a sedimentation procedure (Weichenhan, 1991).

**RESULTS**

**P. stutzeri JM300 restricts DNA**

To test for DNA restriction in strain JM300 (reference strain of genomovar 8; Rossello et al., 1991) we used electroporation

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains and plasmids</th>
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<tr>
<td><strong>Strain/plasmid</strong></td>
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<tr>
<td><strong>P. stutzeri strains</strong></td>
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<tr>
<td>JM300</td>
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<tr>
<td>JB12</td>
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<tr>
<td>CB1–CB6</td>
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<tr>
<td>CB61</td>
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<tr>
<td>ATCC 17587</td>
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<tr>
<td><strong>E. coli strain</strong></td>
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<tr>
<td>SF8</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pUCPKS</td>
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<tr>
<td>pNS1</td>
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<tr>
<td>pKT210</td>
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<tr>
<td>pUCP7-4</td>
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<tr>
<td>pCB25</td>
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<td>pCB20</td>
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<td>pPM1</td>
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to introduce duplex circular DNA of the *E. coli*–*P. stutzeri* shuttle vector pUCPT7-4 which confers tetracycline resistance (Tc\(^R\)) into the cells. When the plasmid was isolated from JM300, the Tc\(^R\) transformation frequency (2 × 10\(^{-4}\) at 50 ng DNA per assay) was consistently about 100-fold higher than that of plasmid prepared from *E. coli* SF8. A similar result was also obtained when the plasmid was prepared from an r\(_{K12}\), m\(_{K12}\) strain of *E. coli* (DH5\(\chi\)). Plasmids pCB20 and pCB25 (Fig. 1) consisting of the pUCPKS vector with inserts of chromosomal DNA from JM300 gave about 100-fold higher electroporation frequencies when the plasmids came from JM300 instead of SF8. In these experiments selection was for the plasmid marker ampicillin resistance (Ap\(^R\)). In the case of another *P. stutzeri* strain from our collection (ATCC 17587; reference strain of genomovar 4; Rossello *et al.*, 1991), the frequency of transformation with plasmid DNA through electroporation was independent of the DNA source (*E. coli*, *P. stutzeri* JM300 or *P. stutzeri* ATCC 17587) and the frequency was as high as that obtained with JM300 as recipient. Moreover, plasmid DNA isolated from strain ATCC 17587 was at least 20-fold more effective in electrocompetent cells of ATCC 17587 than of JM300. These results indicate that JM300 restricts DNA and that strain ATCC 17587 is an isolate (from clinical material; Stanier *et al.*, 1966) having no restriction system comparable to that present in strain JM300.

**Restriction during natural transformation**

We examined the influence of restriction during natural transformation of JM300 with cloned chromosomal DNA. We used plasmid pCB25 (Fig. 1) containing his\(_X\)::Gm\(^R\) (from the JM300 derivative JB12) and selection was for Gm\(^R\). The transformation frequency with JM300 DNA at 3.3 μg ml\(^{-1}\) (5.5 ± 1.7 × 10\(^{-6}\); \(n = 4\)) was about 20-fold higher than that with *E. coli* SF8 DNA (2.6 ± 1.8 × 10\(^{-7}\); \(n = 4\)). The majority of the transformants had lost the chromosomal his\(_X\)^+ by allelic exchange with his\(_X\)::Gm\(^R\) as indicated by their His\(^-\) phenotype. Only 22% remained His\(^+\) and were Ap\(^R\) (29 from 130 tested), suggesting that they contained intact plasmid reconstituted by annealing of single strands having opposite polarity and the breakpoint at different locations (Canosi *et al.*, 1981). Perhaps restriction-sensitive annealing products lead to lower transformation frequencies. We repeated the experiment with plasmid DNA linearized by *XbaI* (restriction site in the vector DNA) to minimize plasmid reconstitution. Linear pCB25 produced again an about 15-fold higher Gm\(^R\) transformation frequency when the DNA was from JM300 (9.6 ± 4.4 × 10\(^{-6}\); \(n = 4\)) compared to DNA from SF8 (6.5 ± 3.1 × 10\(^{-7}\); \(n = 4\)). Transformants containing a reconstituted plasmid were rare (≤4%). Similar results were obtained with pCB20 in which the his\(_X\)::Gm\(^R\) is located approximately in the middle of the insert (Fig. 1). These data suggest that restriction acts during natural transformation of JM300. Restriction levels seen in these initial experiments were somewhat lower than those in later series of experiments in which restriction factors were routinely about 40 (see Tables 2 and 3).

In the above experiments the decreased level of transformant formation was taken as a measure of restriction. However, decreased transformation could also result if the DNA isolated from *E. coli* was hindered from entering cells, perhaps as a consequence of its methylation status. In order to exclude this uncertainty it was necessary to have a restriction-deficient mutant of *P. stutzeri* JM300 to compare its level of transformation with that of the wild-type using the same donor DNA.

**Isolation and characterization of restriction-deficient mutants**

Restriction-deficient (r\(^-\)) mutants were isolated from a culture of strain JM300 after mutagenesis by nitrosoguanidine. We enriched the culture for r\(^-\) mutants by three subsequent electroporation steps with non-modified shuttle vector plasmid DNA (prepared from *E. coli* SF8) able to replicate in *P. stutzeri* and carrying different antibiotic resistance markers. It was assumed that lack of restriction would increase establishment of non-modified plasmids. For the first electroporation we used pUCPKS (Ap\(^R\)). From about 5000 transformed clones selected on Ap plates an electrocompetent cell suspension was electroporated with pNS1 (Gm\(^R\), Km\(^R\)). About 5000 Gm\(^R\) transformed clones were selected and used for the third electroporation step with plasmid pKT210 (Cm\(^R\), Str\(^R\); selection: Cm\(^R\)). Then individual transformant clones were screened for lack of restriction by testing for increased levels of natural transformation with a fourth non-modified plasmid conferring Tc\(^R\) (pUCPT7-4). The screening was performed on agar plates where cells were streaked on a small area of about 1 cm\(^2\), then DNA (5 μl of a 60 μg ml\(^{-1}\) solution) was spotted on the area and after 6 h at 37°C, selection for Tc\(^R\) transformants was applied by spraying the plate with a tetracycline solution. Among 200 screened strains, 12 strains (6%) with decreased restriction efficiency were identified. Six of these strains (with similar growth and competence as

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**Fig. 1.** Restriction map of the chromosomal region flanking the his\(_X\) gene (arrow, indicating length and direction of transcription) in *P. stutzeri* JM300. The PsI restriction sites are marked by short vertical lines. Strains JB12 and CB61 have a Gm\(^R\) cassette inserted in the NdeI restriction site of his\(_X\) as indicated. The various fragments of the chromosomal region present in plasmids pCB20, pCB25 and pPM1 are shown.

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the parental strain) were further characterized after plasmid-free isolates were obtained (strains CB1 to CB6).

Electroporation of strain CB6 with pUCPT7-4 showed that the level of transformation was independent of the source of DNA (Table 2). Similar results were obtained with CB1 to CB5. To test whether the loss of restriction was accompanied by a loss of DNA modification, pUCPT7-4 was isolated from one transformant of each r- mutant and used for electroporation of strain JM300. The level of transformation by plasmid DNA (50 ng per assay) from CB6 (2.7 ± 1.1 × 10^-4; n = 3) was as high as that obtained by the plasmid isolated from JM300 (1.8 ± 1.1 × 10^-4; n = 5), whereas transformation by control DNA (plasmid from E. coli SF8) was about 100-fold lower (1.7 ± 0.5 × 10^-6; n = 4). Results with plasmids from CB1 to CB5 were similar. These data suggest that the mutants CB1 to CB6 had lost their restriction capacity but retained their ability for DNA modification.

Defective restriction renders natural transformation independent of the source of transforming DNA

Natural transformation of JM300 with pCB25 (selection for GmR) was about 43-fold lower when the plasmid came from SF8 and not from JM300 (Table 2). In the restriction-defective mutant CB6 (and similarly in CB1 to CB5) the ratio of transformation frequencies with the two DNA preparations was close to one and at the level seen in JM300 with properly modified DNA. This suggests that (i) the restriction was eliminated in the mutants and (ii) the uptake of P. stutzeri DNA replicated in E. coli SF8 was as efficient as that of DNA replicated in P. stutzeri JM300.

Since during natural transformation only a single strand enters the cytoplasm (Dubnau, 1999), which is normally not a target of restriction enzymes, we considered the possibility that due to the rather high plasmid DNA concentration in these assays, frequently strands of the transforming DNA with opposite polarity would enter a cell and after annealing, might be attacked by restriction enzymes. Such a situation has been described previously in natural transformation of B. subtilis with phage DNA (transfection) where strong restriction of the phage DNA was observed (Bron et al., 1980). At low DNA concentrations, when cells take up at best only one strand, restriction should be minimal if annealed DNA was the major substrate for restriction. This was not the case. When derivatives of JM300 and CB6 having a chromosomal hisX::GmR gene (strains JB12 and CB61, respectively) were transformed (selection: His+) with pPM1 (carrying hisX+) isolated from SF8, the approximately 50-fold lower transformation of JB12 compared to CB61 seen at high DNA concentrations (33 μg ml^-1) was also seen at concentrations down to 0.03 μg ml^-1 (Fig. 2), which corresponds to about 0.1 plasmid per recipient cell (Fig. 2). This result indicates that restriction during natural transformation with cloned chromosomal DNA cannot be explained by restriction of annealed donor DNA strands.

Restriction during natural transformation with single-stranded DNA

P. stutzeri JM300 is transformable by single-stranded DNA and this requires the same cellular components as those required for transformation with duplex DNA, including type IV pili and the ComA protein, which forms the presumptive DNA pore in the cytoplasmic membrane (Meier et al., 2002). For transformation we used the single-stranded DNA isolated from CsCl-purified phage fd containing the cloned antisense strand of P. stutzeri JM300 hisX+ (on a 2.18 kb PsrI fragment; Fig. 1) after replication in E. coli and in addition, the duplex replicative form of the phage DNA (pPM1) prepared in E. coli. The transformation frequency (selection: His+) of strain CB61 with single-stranded DNA was significantly higher than that of JB12 (15-fold; Table 3). With linearized duplex DNA (pPM1) the transformation frequency of CB61 was 44-fold higher than that of JB12, which corresponds with the data of Table 2 and Fig. 2. These experiments show that DNA restriction in P. stutzeri JM300 acts also during transformation with single-stranded DNA.

### Table 2. Electroporation and natural transformation of P. stutzeri JM300 and strain CB6 with pUCPT7-4 or pUCP25 DNA, respectively, isolated from P. stutzeri JM300 or E. coli SF8

Electroporation was with 50 ng DNA per assay and natural transformation was with 3-3 μg DNA ml^-1. The data are means from two independent experiments.

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>Transformation frequency after electroporation*</th>
<th>Transformation frequency after natural transformation†</th>
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<tbody>
<tr>
<td></td>
<td>A†</td>
<td>B§</td>
</tr>
<tr>
<td>JM300</td>
<td>1.8 ± 1.1 × 10^-4</td>
<td>1.7 ± 1.1 × 10^-6</td>
</tr>
<tr>
<td>CB6</td>
<td>8.4 ± 6.6 × 10^-4</td>
<td>8.6 ± 4.4 × 10^-4</td>
</tr>
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</table>

*pUCPT7-4 DNA was used. Selection was for TcR.
†pUCP25 DNA was used. Selection was for GmR. The GmR transformants included all His- Ap5.
‡DNA isolated from P. stutzeri JM300.
§DNA isolated from E. coli SF8.
DNA restriction during natural transformation of *P. stutzeri* JM300 decreased the formation of transformants by cloned chromosomal DNA and plasmids replicated in *E. coli* by about 40-fold, and was somewhat weaker than restriction during electroporation (about a 100-fold reduction), which introduces duplex DNA into cells. These results correspond with previous observations made with JM300 and different shuttle vector plasmids (Lorenz *et al.*, 1998). In the r− mutants isolated from strain JM300, restriction of transforming duplex and single-stranded DNA was abolished. The nature of the RM system of JM300 is not yet known. Preliminary experiments with cell-free extracts aimed at observing restriction of DNA *in vitro* were not successful (our unpublished results). However, as pointed out by Wilson & Murray (1991), *in vitro* assays for restriction enzymes have limitations, and bacteria scored as negative can in fact be positive. As all six r− mutants still modified DNA, the RM system in JM300 is probably of type II (Bickle & Krüger, 1993). In contrast, of the r− mutants isolated from bacteria with type I or type III RM systems, generally about half were also modification-deficient (Meselson *et al.*, 1972). The natural isolate of *P. stutzeri*, strain ATCC 17587, was identified here to have no corresponding RM system.

It has been stated that restriction does not act during natural transformation (Majewski, 2001) because a single strand (which is not a normal substrate of restriction endonucleases) is delivered into the cell and subsequently recombines with the chromosome, giving a hemimethylated duplex, which is normally not restricted either. Transformation studies in species (*B. subtilis*, *H. influenzae* and *S. pneumoniae*) in which restricting and non-restricting isogenic strains were available supported this view (Bron *et al.*, 1980; Stuy, 1976; Lacks & Springhorn, 1984), while transfection was strongly decreased by restriction in *H. influenzae* and *B. subtilis*. The latter presumably resulted from cytoplasmic annealing of phage DNA strands leading to restriction-sensitive non-modified duplexes. On the other hand, it was found later that the vast majority of restriction endonucleases also cleave non-modified single-stranded DNA, although at a lower rate than corresponding duplexes (Nishigaki *et al.*, 1985; Horiiuchi & Zinder, 1975; Blakesley & Wells, 1975). This opens the general possibility of restriction during natural transformation. Interestingly, in *B. subtilis* the *Bsp*I restriction enzyme cleaves purified single-stranded phage DNA *in vitro* quite effectively but *in vivo* it does not reduce the transfecting activity of purified phage single strands in marker rescue assays employing coinfection with modified phage (Bron *et al.*, 1980). It was concluded that the complexing of the single strand with single-strand binding protein upon entering the cytoplasm (see Lacks, 1999) protects the DNA from restriction. This protection gets lost upon annealing with a non-modified complementary strand. A different situation is observed in *S. pneumoniae* harbouring the DpsII RM system. These cells express the single-strand-specific modification methyltransferase during

**DISCUSSION**

**Table 3.** Natural transformation frequencies of JM300 derivatives JB12 (*r+ hisX::GmR*) and CB61 (*r− hisX::GmR*) with single-stranded (ss) DNA and duplex (ds) DNA of pPM1 replicated in *E. coli*.

Single-stranded DNA and duplex DNA were used at 33 μg ml⁻¹. The *his+* reversion frequency of the strains was ≤4·7×10⁻¹⁰. All transformants were Ap sensitive. The data are given with standard deviation.

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>ss DNA*</th>
<th>Relative value</th>
<th>n</th>
<th>ds DNA†</th>
<th>Relative value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB12</td>
<td>6·0±3·1×10⁻⁹</td>
<td>1</td>
<td>5</td>
<td>3·4±0·1×10⁻⁸</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>CB61</td>
<td>9·4±7·6×10⁻⁸</td>
<td>15·7</td>
<td>6</td>
<td>1·5±0·6×10⁻⁶</td>
<td>44·1</td>
<td>6</td>
</tr>
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</table>

*hisX*+ anti-sense strand. A t-test indicated significantly higher transformation frequencies in CB61 than in JB12 (*P*<0·05).

†pPM1 *hisX*+, linearized by *Scal*.
competence leading to effective protection of transforming DNA (Lacks et al., 2000).

At what stage in natural transformation of *P. stutzeri* could restriction act? Presynaptic and postsynaptic attacks of restriction endonucleases may be considered. Perhaps different from *B. subtilis*, in *P. stutzeri* the DNA binding protein associating with the single-stranded DNA does not protect DNA against restriction. This could largely eliminate its transforming potential if these sites are frequently present in the DNA. A postsynaptic attack could occur if the non-modified strand was integrated into hemimethylated genomic regions in a way producing duplex regions lacking methylation. The resulting double-strand break would be lethal in the absence of repair and would thus eliminate the transformant. Hemimethylated DNA is expected to be transient and close behind the replication fork. A double-strand break in this region can be repaired by recBCD-dependent recombination using the other replicated duplex as homologous DNA (Kuzminov, 1999). Since the repair involves degradation at DNA ends the genetic marker may often be lost. The 40-fold reduction in natural transformation would require that DNA is almost always incorporated at hemimethylated sites and in a way leading to replacement of the methylated strand. This is difficult to understand.

Any barrier that will limit intra- and interspecific genetic exchange in prokaryotes will contribute to sexual isolation and thereby will foster speciation (Majewski, 2001). In contrast to observations in *B. subtilis, H. influenzae* and *S. pneumoniae*, our data with *P. stutzeri* JM300 and ATCC 17587 suggest that restriction can contribute to sexual isolation among transformable bacteria. In a recent study on intra- and interspecific transformation in *Pseudomonas*, it was observed that strain ATCC 17587 described here as having no RM system was the strain least isolated from other species (including *Pseudomonas alcaligenes* and *Pseudomonas mendocina*) and also from members of other genomic groups of *P. stutzeri* (including members of six genomovars), whereas strain JM300 was most strongly isolated from all strains, even from the strain most closely related to JM300 in that study (Lorenz & Sikorski, 2000). Moreover, the sexual isolation of JM300, when acting as a recipient for DNA from the other strains, was much stronger than expected on the basis of the nucleotide sequence divergence to the different donor DNAs. The authors suspected that besides sequence divergence and a different level of DNA uptake competence, other not yet identified genetic factors could contribute to the sexual isolation of *P. stutzeri* JM300. The restriction system of JM300 described here could be such a factor. Presently, only five RM systems have been detected in *P. stutzeri* (see http://rebase.neb.com), and it is not yet known how frequent and diverse RM systems are among the over 500 members of that species identified by molecular methods (Sikorski et al., 2002a, b). Several authors have pointed out that RM systems can contribute to an increase of genetic diversity in prokaryotes by allowing natural genetic engineering involving homologous and illegitimate recombination processes and by exerting certain selection pressure on the DNA sequence (Arber, 1991; Kusano et al., 1997; McKane & Milkman, 1995; Rocha et al., 2001). The data of this study add the notion that RM systems can contribute to speciation also by producing sexual isolation and thereby allowing the free divergence of the isolated lineage.

**ACKNOWLEDGEMENTS**

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DNA restriction in *P. stutzeri* transformation


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