JOURNAL OF BACTERIOLOGY, June 1996, p. 3374–3376 Vol. 178, No. 11
0021-9193/96/$04.00+0

The *sre* Gene (ORF469) Encodes a Site-Specific Recombinase Responsible for Integration of the R4 Phage Genome

MANABU MATSUURA,[1] TAKAHIKO NOGUCHI,[1] DAISUKE YAMAGUCHI,[1] TOKUJIRO AIDA,[1] MUNEHIKO ASAYAMA,[1] HIDEO TAKAHASHI,[2] AND MAKOTO SHIRAI[1]*

Division of Biotechnology, School of Agriculture, Ibaraki University, Ami, Inashiki, Ibaraki 300-03,[1] and Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113,[2] Japan

Received 27 December 1995/Accepted 3 April 1996

The *sre* gene (ORF469) of the R4 phage encodes a protein similar to the resolvase-DNA invertase family proteins. Insertional gene disruption of *sre* prevented a lysogen from entering the lytic cycle, implying that Sre protein is a site-specific recombinase needed for excision of the R4 prophage genome (M. Matsuura, T. Noguchi, T. Aida, M. Asayama, H. Takahashi, and M. Shirai, J. Gen. Appl. Microbiol. 41:53–61, 1995). To determine whether this *sre* gene is also necessary for the integration reaction, we studied its function by integration plasmid analysis. When deletions, frameshifts, and site-directed mutations that caused an amino acid substitution of Ser-17 for Ala were introduced into the *sre* structural gene, transformation efficiency of *Streptomyces parvulus* 2297 with these plasmid DNAs was severely reduced. However, an adenine insertion just before the possible initiation codon of the *sre* gene did not significantly decrease the efficiency. These data suggest that the Sre protein is a site-specific recombinase responsible for integration of the R4 phage genome.

Streptomyces strains are important organisms because they produce a variety of useful secondary metabolites, for example, antibiotics, antitumor reagents, and immunosuppressors (3). R4 is an actinophage that has been studied in depth from the viewpoint of its practical application (12–17). To improve the genetic engineering of *Streptomyces* strains, site-specific recombination of this R4 phage has been studied (11, 21). Our data suggested that the ORF469 gene (*sre*) (DDBJ accession no. D38173), which was found adjacent to the *attP* recombination site of the R4 phage genome, is essential for excision of the prophage genome and probably encodes a site-specific recombinase responsible for the excisive recombination (11). The protein encoded by ORF469 falls not into the integrase family but into the resolvase-DNA invertase family and is highly similar to Tn*2501* resolvase and SpoIVCA of *Bacillus subtilis* (11). The ORF469 protein and SpoIVCA have additional C-terminal halves consisting of about 300 amino acids that typical resolvases do not have, and this kind of recombinase has been also found in *Anabaena* spp. (XisF) (2, 20). In this study, we showed that the protein encoded by the ORF469 gene (*sre*) is a site-specific recombinase responsible for integration of the R4 phage genome into the *Streptomyces parvulus* 2297 chromosome.

Construction of integration plasmids. To introduce a thiostrepton resistance gene (*tsr*) (6) as a selection marker into an integration plasmid, a 1.1-kb *Bcl*I fragment from pIJ702 containing the *tsr* gene was inserted by blunt-end ligation into the *Sma*I site of pAT95 (the *Sma*I site was in the multicloning site of the pUC18 vector), which contains a 13-kb *Bcl*I fragment carrying the ORF469 gene and the *attP* recombination site from the R4 phage genome (11), to generate pAT96L (data not shown). A 5.6-kb *Eco*RI-*Sca*I fragment carrying *tsr*, ORF469 (*sre*), and *attP* was isolated from pAT96L and inserted between the *Sma*I and *Eco*RI sites of pUC118 to create pAT97 (8.8 kb) (Fig. 1A). To delete the 4.5-kb fragment of the subcloned phage DNA from the upstream region, pAT97 was linearized by *Pst*I and *Xba*I digestion (both sites in the multicloning site of pUC118) and then unidirectionally deleted with exonuclease III and mung bean nuclease (19). Deleted inserts of four clones (pAT97D1, pAT97D2, pAT97D3, and pAT97D4) are shown in Fig. 1C. Plasmids pATD-M and pATD-S, which contain the defective ORF469 gene, were constructed by deletion of a 0.7-kb *Mlu*I fragment and a 1.6-kb *Spl*I fragment from pAT97D4, respectively (Fig. 1C).

Plasmid pAT98 (6.0 kb) for a site-directed mutation of ORF469 was constructed by ligating a 1.9-kb *Hin*dIII-*Kpn*I fragment carrying ORF469 (*sre*) and *attP* from pAT97D4 into the *Hin*dIII and *Kpn*I sites of BluescriptIIKS+ and then ligating a 1.1-kb *Eco*RI-*Hin*dIII fragment carrying *tsr* from pTSR-1 into the *Eco*RI-*Hin*dIII sites (Fig. 1B). Plasmid pTSR-1 was constructed by inserting a 1.1-kb *tsr Bcl*I fragment of pIJ702 into the *Bam*HI site of pUC118.

Plasmids pMFS1, pMFS2, pMFS3, and pMAS2 were derived from pAT98. Each has a site-directed mutation in the ORF469 gene (*sre*) (Table 1). These mutations were introduced as described by Kunkel (10) by using the site-directed mutation kit Mutan-K (Takara, Kyoto, Japan) according to its instruction manual. A single-stranded DNA of M13HS0.5 was used as a template. M13HS0.5 was constructed by inserting a 0.5-kb *Hin*dIII-*Sal*I fragment of pATD2 carrying the ORF469 gene (11) between the *Hin*dIII and *Sal*I sites of M13mp18. The 0.5-kb fragment contains a 5′ end of the ORF469 structural gene. FS1 primer (25-mer; CTCTGCGGGTGA**A**TGAATCG AGGGG), FS2 primer (25-mer; CTGCGGGTGATG**A**AATC GAGGGGGG), FS3 primer (24-mer; GCCTCGGCATG**A**GA GGTGGGGCAG), and AAS2 primer (25-mer; CTACGTCC GAATC**GC**CCTGGACCGC) (the inserted or changed bases are in boldface) were used for the mutations in pMFS1, pMFS2, pMFS3, and pMAS2, respectively. After confirmation of the mutated sequences, 0.5-kb *Hin*dIII-*Sal*I fragments of

* Corresponding author. Mailing address: Division of Biotechnology, School of Agriculture, Ibaraki University, Ami, Inashiki, Ibaraki 300-03, Japan. Phone: (81)298-88-8652. Fax: (81)298-88-8653. Electronic mail address: shirai@agr.ibaraki.ac.jp.

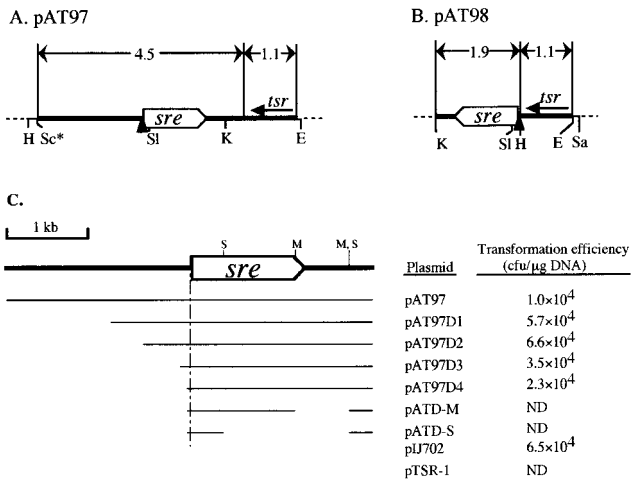

FIG. 1. Construction of integration plasmids and effect of deletion on transformation efficiency. (A and B) Integration plasmids pAT97 and pAT98, respectively. Thick horizontal lines show two exogenous DNA fragments that were inserted into pUC118 (A) or pBlueScript II KS+ (B). The *sre* gene is indicated by unfilled arrow boxes. Solid upward triangles indicate the positions of the *attP* recombination site. The thiostrepton resistance gene (*tsr*) is shown by horizontal arrows. Thin horizontal lines indicate short DNA sequences derived from the multicloning sites of cloning vectors, and dotted lines indicate parts of cloning vectors. Lengths of the inserted fragments are presented in kilobases. (C) Effects of deletion on transformation efficiency. R4 phage-derived fragments of the plasmids are represented by thin horizontal lines, and the dotted vertical line indicates the position of the *attP* recombination site. Symbols for the restriction endonucleases are as follows: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; S, *Spl*I; Sl, *Sal*I; Sa, *Sac*I; and Sc*, *Sca*I cleavage site of the phage DNA fragment ligated with *Sma*I cleavage site of vector. ND, none detected.

each clone were used to replace a 0.2-kb *Hin*dIII-*Sal*I fragment of pAT98.

Integration plasmid analysis. We predicted that the putative ORF469 protein (Sre) was required for site-specific integration of the R4 phage genome into the *S. parvulus* 2297 chromosome as well as the excision of a prophage genome for the following reasons. (i) The predicted amino acid sequence showed homology to those of the other bacterial site-specific recombinases, i.e., TnpR of Tn*2501* and SpoIVCA of *B. subtilis* (11). (ii) The results of the gene disruption study suggested that the protein (Sre) was a site-specific recombinase required for excision of the R4 prophage genome from a lysogen chromosome (11). (iii) It is likely that the reversible site-specific recombination is catalyzed by a site-specific recombinase. As the size of bacteriophage genomes is limited, it would be an advantageous strategy to use a site-specific recombinase in both integrative and excisive reactions. In fact, the Int protein of phage λ acts as a site-specific recombinase in both types of reactions (1, 8, 18).

To test this notion, we analyzed the integration function of ORF469 with plasmids which do not replicate in *Streptomyces* cells. Two such integration plasmids, pAT97 and pAT98, are shown in Fig. 1. If ORF469 encodes a recombinase responsible for the R4 integrative reaction, these plasmids should be integrated into the host chromosome and transform it into being thiostrepton resistant. However, if it does not facilitate recombination, *S. parvulus* 2297 should not be transformed. Protoplasts were prepared and *S. parvulus* 2297 was transformed as described previously (7).

It was suggested that the 4.5-kb *Sca*I-*Bcl*I fragment of pAT97 facilitates recombination between an integration plasmid and a host chromosome. When we used pAT97, *S. parvulus* 2297 was transformed at an efficiency of 1×10^4 CFU/μg of DNA, whereas it was transformed at an efficiency of 6.5×10^4 CFU/μg of DNA with pIJ702, which is a multicopy plasmid replicating in *Streptomyces* cells (Fig. 1C). On the other hand, we did not detect any thiostrepton transformants with pTSR-1, which does not have an R4 phage-derived DNA fragment. These data suggested that the 4.5-kb *Sca*I-*Bcl*I fragment of the R4 phage genome facilitates integrative recombination.

We then showed that the ORF469 structural gene is necessary for the efficient transformation of *S. parvulus* 2297. To locate the region essential for the efficient transformation of *S. parvulus* 2297 with integration plasmids, deletions were introduced into the 4.5-kb *Sca*I-*Bcl*I fragment. Plasmids pAT97D1, pAT97D2, pAT97D3, and pAT97D4 were constructed as a unidirectional deletion series carrying R4 phage-derived inserts of 3.2, 2.8, 2.4, and 2.2 kb, respectively (Fig. 1C). The fact that *S. parvulus* 2297 was transformed as efficiently with these four plasmids as it was with pAT97 showed that there is no important function in the deleted upstream region. In pAT97D4, there is only 31 bp remaining upstream of the *attP* core region. On the other hand, pATD-M and pATD-S, which are derivatives of pAT97D4 (with additional deleted regions) that contain the ORF469 structural gene, did not transform *S. parvulus* 2297 (Fig. 1C). These data support the notion that the ORF469 gene encodes a site-specific recombinase responsible for integration of the R4 phage genome into the host chromosome.

To confirm that these integration plasmids were actually integrated into the chromosomes of *S. parvulus* site specifically, genomic DNAs from three thiostrepton-resistant transformants (two pAT97 derivatives and one pAT97D4 derivative) were Southern hybridized with an *attB* fragment as a probe (21). Furthermore, two plasmid-chromosome junction fragments (*attL* and *attR*) were subcloned from the thiostrepton transformants of pAT97 and pAT97D4, respectively, and sequenced. These analyses confirmed the integration of the plasmids and their site specificity (data not shown). Thus, the transformation efficiency with the integration plasmids indicates the efficiency of the integrative site-specific recombination between the plasmids and the host chromosome.

We showed that the protein encoded by the ORF469 gene was required for site-specific integration by using plasmids with site-directed frameshifts. The ORF469 genes in pMFS1, pMFS2, and pMFS3 have an adenine inserted at various positions (Table 1). In pMFS1 and pMFS2, it was inserted just before and after the putative initiation codon of the ORF469 gene, respectively. In pMFS3, it was inserted just after the second methionine codon (Met-43), which was another candidate initiation codon of the gene (11). *S. parvulus* 2297 was transformed with pMFS1 at an efficiency about one-fifth of that with pAT98, which had the wild-type sequence of the ORF469 gene (Table 1). On the other hand, pMFS2 and

TABLE 1. Effect of site-directed mutation on transformation efficiency

Plasmid	Mutation[a]			Relative transformation efficiency (%)
	Met-1 ATG	Ser-17 AGC	Met-43 ATG	
pAT98				100[b]
pMFS1	AATG			18
pMFS2	ATGA			<0.3
pMFS3			ATGA	<0.3
pMAS2		GCC		<0.3

[a] Only mutated regions are depicted. Inserted and substituted nucleotides are underlined.

[b] Actual transformation efficiency with pAT98, 3.5×10^2 CFU/μg of DNA.

pMFS3 did not transform *S. parvulus* 2297 (Table 1). These data supported the notion that the protein encoded by the ORF469 gene is a site-specific recombinase responsible for the integrative reaction, because only insertions causing frameshifts in the ORF469 structural gene (FS2 and FS3) markedly reduced the efficiency. We considered that the moderate reduction with pMFS1 might have resulted from a lowered transcription or translation rate due to the insertion.

The concept that the ORF469 protein is a member of the resolvase-DNA invertase family was supported by the results of an experiment using a plasmid with a site-directed mutation that caused an amino acid substitution. Plasmid pMAS2 has base substitutions causing an amino acid substitution at position 17 from Ser to Ala (Table 1). This position corresponds to the Ser residue that is thought to be an active center of resolvases and DNA invertases (5, 11). Although we cannot rule out the possibility of a conformational change of the protein due to the substitution that affects its activity, the fact that we could not detect any thiostrepton transformants with pMAS2 suggested that the ORF469 protein is a resolvase-related site-specific recombinase.

The results of these integration plasmid analyses and our published data showed that the ORF469 gene is essential for site-specific integration and excision between the R4 phage genome and the *S. parvulus* 2297 chromosome. Primarily, we showed here that the protein encoded by the gene was a site-specific recombinase responsible for the integrative reaction. On the basis of these findings, we named the ORF469 gene *sre* (for site-specific recombinase).

Stability of the integrated plasmids. The data obtained from the integration plasmid analysis showed that *sre* was essential and sufficient for site-specific integration of the R4 phage genome except for the *attP* recombination site. On the other hand, our earlier data suggested that *sre* was essential for the site-specific excision of the R4 prophage genome. If the factor(s) encoded by the integrated plasmids is also essential and sufficient for the site-specific excision, the stability of these plasmids should be reduced under conditions without selective pressure. To determine whether the integrated plasmids could be efficiently excised, we measured their stability by a replica plating experiment from minus thiostrepton to plus thiostrepton. The spores of transformants with pAT97 and pAT97D4 were isolated from a sporulating agar culture and spread on YS plates (11) without thiostrepton. After 10 days, these master plates were replicated onto YS agar plates with thiostrepton and cultured. Loss of thiostrepton resistance was not observed with either transformant (frequencies of loss, $<6.1 \times 10^{-4}$ and $<9.6 \times 10^{-4}$ for pAT97 and pAT97D4, respectively), implying that these plasmids did not have all the elements required for site-specific excision.

REFERENCES

1. **Abremski, K., and S. Gottesman.** 1982. Purification of the bacteriophage lambda *xis* gene product required for λ excisive recombination. J. Biol. Chem. **257:**9658–9662.
2. **Carrasco, C. D., K. S. Ramaswamy, T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena xisF* gene encodes a developmentally regulated site-specific recombinase. Genes Dev. **8:**74–83.
3. **Goodfellow, M., S. T. Williams, and M. Mordarski.** 1988. Actinomycetes in biotechnology. Academic Press, San Diego, Calif.
4. **Hatfull, G. F., and N. D. F. Grindley.** 1986. Analysis of γδ resolvase mutants *in vitro*: evidence for an interaction between serine-10 of resolvase and site I of *res*. Proc. Natl. Acad. Sci. USA **83:**5429–5433.
5. **Hatfull, G. F., and N. D. F. Grindley.** 1988. Resolvases and DNA-invertases: a family of enzymes active in site-specific recombination, p. 357–396. *In* R. Kucherlapati and G. R. Smith (ed.), Genetic recombination. American Society for Microbiology, Washington, D.C.
6. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*. The John Innes Foundation, Norwich, England.
7. **Isogai, T., H. Takahashi, and H. Saito.** 1980. High-frequency protoplast-transfection of *Streptomyces parvulus* 2297 with actinophage R4 DNA. Agric. Biol. Chem. **44:**2425–2428.
8. **Kikuchi, Y., and H. Nash.** 1979. Nicking-closing activity associated with bacteriophage λ *int* gene product. Proc. Natl. Acad. Sci. USA **76:**3760–3764.
9. **Kilby, N. J., M. R. Snaith, and J. A. H. Murray.** 1993. Site-specific recombinases: tools for genome engineering. Trends Genet. **9:**413–421.
10. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phonotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
11. **Matsuura, M., T. Noguchi, T. Aida, M. Asayama, H. Takahashi, and M. Shirai.** 1995. A gene essential for the site-specific excision of actinophage R4 genome from the chromosome of a lysogen. J. Gen. Appl. Microbiol. **41:**53–61.
12. **Mitsui, H., and H. Takahashi.** 1992. Cohesive single-stranded ends of *Streptomyces* temperate bacteriophage R4. Mol. Gen. Genet. **231:**360–362.
13. **Mitsui, H., and H. Takahashi.** 1992. Delimitation of cohesive ends site (*cos*) of *Streptomyces* temperate bacteriophage R4. Biochem. Biophys. Res. Commun. **186:**1663–1668.
14. **Morino, T., T. Isogai, H. Kojima, H. Takahashi, and H. Saito.** 1983. Cleavage analysis of actinophage R4 and its deletion mutants. Agric. Biol. Chem. **47:**1873–1878.
15. **Morino, T., T. Isogai, H. Takahashi, and H. Saito.** 1984. Construction of phage vectors in *Streptomyces*: introduction of the thiostrepton resistant (*tsr*) gene into R4 phage. Agric. Biol. Chem. **48:**1985–1990.
16. **Morino, T., K. Takagi, T. Nakamura, T. Takita, H. Saito, and H. Takahashi.** 1986. Studies of cosmid transfection in *Streptomyces lividans* and *Streptomyces parvulus*. Agric. Biol. Chem. **50:**2493–2497.
17. **Morino, T., H. Takahashi, and H. Saito.** 1985. Construction and characterization of a cosmid of *Streptomyces lividans*. Mol. Gen. Genet. **198:**228–233.
18. **Nash, H. A., and C. A. Robertson.** 1981. Purification and properties of the *Escherichia coli* protein factor required for λ integrative recombination. J. Biol. Chem. **256:**9246–9253.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Sato, T., Y. Samori, and Y. Kobayashi.** 1990. The *cisA* cistron of *Bacillus subtilis* sporulation gene *spoIVCA* encodes a protein homologous to a site-specific recombinase. J. Bacteriol. **172:**1092–1098.
21. **Shirai, M., H. Nara, A. Sato, T. Aida, and H. Takahashi.** 1991. Site-specific integration of the actinophage R4 genome into the chromosome of *Streptomyces parvulus* upon lysogenization. J. Bacteriol. **173:**4237–4239.