DNA RESEARCH 5, 327–334 (1999)

A Novel Plasmid Recombination Mechanism of the Marine Cyanobacterium *Synechococcus* sp. PCC7002

Hideo AKIYAMA,[1,*] Shozo KANAI,[1] Masahiko HIRANO,[1] and Hitoshi MIYASAKA[2]

Biological Sciences Department, Toray Research Center, Inc., 1111 Tebiro, Kamakura, Kanagawa 248-8555, Japan[1] *and Kansai Electric Power Co., Inc., Technical Research Center, 3-11-20 Nakoji, Amagasaki, Hyogo 661-0974, Japan*[2]

(Received 29 June 1998; revised 7 July 1998)

Abstract

We describe a novel mechanism of site-specific recombination in the unicellular marine cyanobacterium *Synechococcus* sp. PCC7002. The specific recombination sites on the smallest plasmid pAQ1 were localized by studying the properties of pAQ1-derived shuttle-vectors. We found that a palindromic element, the core sequence of which is G(G/A)CGATCGCC, functions as a resolution site for site-specific plasmid recombination. Furthermore, site-directed mutagenesis analysis of the element show that the site-specific recombination in the cyanobacterium requires sequence specificity, symmetry in the core sequence and, in part, the spacing between the elements. Interestingly, this element is over-represented not only in pAQ1 and in the genome of the cyanobacterium, but also in the accumulated cyanobacterial sequences from *Synechococcus* sp. PCC6301, PCC7942, *vulcanus* and *Synechocystis* sp. PCC6803 within GenBank and EMBL databases. Thus, these findings strongly suggest that the site-specific recombination mechanism based on the palindromic element should be common in these cyanobacteria.

Key words: plasmid pAQ1; palindromic element; recombination site; pAQJ4; *Synechococcus* sp. PCC7002

1. Introduction

Although several cyanobacterial host-vector systems have been developed, shuttle-vectors based on endogenous cyanobacterial plasmids sometimes cause low frequencies of transformation and lose rapidly their selection markers in the transformants.[1–3] It is considered that the regions that are homologous between the introducing vector and the endogenous plasmid cause to recombine, resulting in the loss of selection markers. In general, recombination events in cyanobacterial genomes occur frequently and hence have played an important part in the exchange or integration of foreign genes. However, a comprehensive understanding of the recombination mechanism in cyanobacterial genome or plasmids is still unknown. Only a little is known about structural plasmid instability and plasmid replication in cyanobacteria.[4–6]

The unicellular cyanobacterium *Synechococcus* sp. PCC7002 (*Agmenellum quadruplicatum* strain PR-6) is a coastal species which is readily transformable by plasmids and genomic DNA. It contains at least six plasmids, ranging in size from 4.5 kb to 112 kb,[7] and we have previously characterized the smallest plasmid pAQ1[8] (Fig. 1). The plasmid pAQ1 has at least four open reading frames (ORFs), ORF943, ORF93, ORF71 and ORF64, and a putative replication origin (*ori*) region similar to that of prokaryotic plasmids.[9] As shown in Fig. 1, it contains eight palindromic elements, the core sequence is G(G/A)CGATCGCC, which is over-represented in plasmid pAQ1 but its function is unknown. In this paper, we describe the palindromic element in pAQ1 functions as a specific recombination signal sequence between exogenous and/or endogenous plasmids. In addition, the element is over-represented not only in known sequences from *Synechococcus* sp. PCC7002, but also from *Synechococcus* sp. PCC6301, PCC7942, *vulucanus* and *Synechocystis* sp. PCC6803, suggesting that the site-specific recombination mechanism based on the palindromic element should be common in these cyanobacteria.

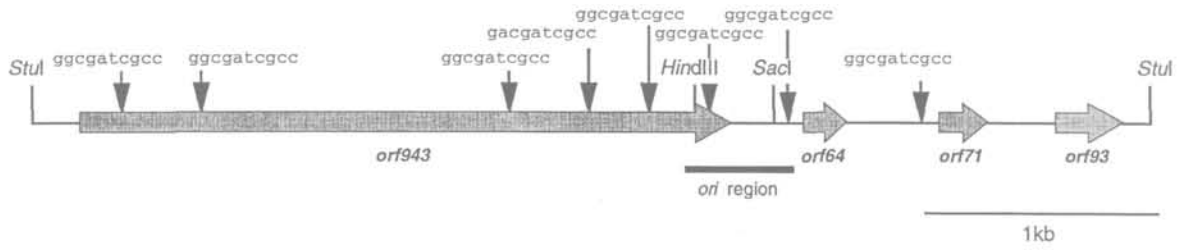
2. Materials and Methods

2.1. Enzymes, chemicals and strains

Restriction endonucleases and other enzymes were purchased from Takara Shuzo (Kyoto) or Amersham Pharmacia Biotech (Uppsala, Sweden). The DNA sequencing kits were from Perkin Elmer (Norwalk, CT). All other chemicals were of the highest commercial purity

Communicated by Masahiro Sugiura

* To whom correspondence should be addressed. Tel. +81-467-32-9962, Fax. +8-467-32-0414, E-mail: Hideo_Akiyama@trc.toray.co.jp



sequence (core;GgCGATCGCC)	location (core sequence)	region
...aaa GGCGATCGCC ccc...	355 - 364	ORF943
...ccg GGCGATCGCC ttc...	726 - 735	ORF943
...taa GGCGATCGCC cgt...	2046 - 2055	ORF943
...cct GACGATCGCC agt...	2365 - 2374	ORF943
...aca GGCGATCGCT ctc...	2627 - 2636	ORF943
...tga GGCGATCGCC cct...	2988 - 2907	ORF943
...cgg GGCGATCGCC ctt...	3224 - 3233	non coding
...ggg GGCGATCGCC cct...	3823 - 3832	non coding

Figure 1. Structure of plasmid pAQ1 from *Synechococcus* sp. PCC7002. The nucleotide residue number begins with the first base of the unique *Stu* I site. The unique *Hin*dIII and *Stu* I sites and four open reading frames (ORFs) are also shown. The putative replication origin (*ori*) region and a family of 8 palindromic sequences G(G/A)CGATCGCC are marked with bold line and arrows, respectively. The nucleotide position and location of palindromic sequences and sequence surrounding the element are also shown.

available.

The cyanobacterial strain *Synechococcus* sp. PCC7002 (*Agmenellum quadruplicatum* PR-6), obtained from the American Type Culture Collection (ATCC 27264), was maintained and cultured under photoautotrophic conditions at 32°C in medium A.[10] The *Escherichia coli* strain JM109 purchased from Takara Shuzo was grown in LB medium.[11]

2.2. *Construction of pAQ1-derived vectors*

Two series of shuttle-vectors were constructed to investigate the presence and location of specific recombination sites on pAQ1. The plasmid pAQ1 was joined with pUC19 at their unique restriction *Hin*dIII sites for the construction of the first series of vectors, named pAQJ1 (Fig. 2a). Similarly, pAQ1 digested at its unique restriction *Stu* I site and was cloned into blunted *Hin*dIII sites on pUC19 to create pAQJ2. Cloning the *Stu* I-*Eco*RI fragment from pAQJ1 into *Eco*RI and blunted *Hin*dIII sites on pUC19 produced a new vector, pAQJ3. After partial digestion with *Sac* I and religation to remove about 2 kb of excess from pAQJ2, the resulting vector, pAQJ4, was subjected to *Eco*RI digestion to introduce synthetic multi-cloning sites as diagrammed.

For preparation of the second series of ORF-deleted vectors, pAQJ-D, the nested deletion method[12] and polymerase chain reaction (PCR) technique were used. The resulting clones were identified by a combination of agarose gel electrophoresis and DNA sequence analysis.

2.3. *Construction of series of palindromic element-containing (PEC) vectors*

The following three sets of oligonucleotides that included a restriction *Sal* I site between two palindromic elements (in bold and underline typeface) were synthesized using an ABI 394-5 DNA synthesizer: 5′-AAT TCT **GGC GAT CGC C**TT TGT CGA CGA A**GG CGA TCG CC**G-3′ for PE(WT:GG) sense primer, and 5′-GAT CC**G GCG ATC GCC** TTC GTC GAC AAA **GGC GAT CGC C**AG-3′ for PE(WT:GG) antisense primer; 5′-AAT TCT **GGC AAT CGC C**TT TGT CGA CGA A**GG CGA TCG CC**G-3′ for PE(AG) sense primer and 5′-GAT CC**G GCG ATC GCC** TTC GTC GAC AAA **GGC GAT TGC C**AG-3′ for PE(AG) antisense primer; 5′-AAT TCT **GGC AAT CGC C**TT TGT CGA CGA A**GG CAA TCG CC**G-3′ for PE(AA) sense primer, and 5′-GAT CC**G GCG ATT GCC** TTC GTC GAC AAA **GGC GAT TGC C**AG-3′ for PE(AA) antisense primer (double underline indicates nucleotide substitution). Each set of oligonucleotides was annealed and inserted into pAQJ4-MCS *Eco*RI and *Bam*HI sites to create PEC(WT:GG), PEC(AG) and PEC(AA), respectively. Cloning palindromic elements containing a Kanamycin Resistance (Km^r) GenBlock (Amersham Pharmacia Biotech) at their *Sal* I sites also provided new vectors, Km + PEC(WT:GG), Km + PEC(AG) and Km + PEC(AA). All vectors were reconfirmed by DNA sequence analysis using an ABI 373S DNA sequencer (Fig. 2b).

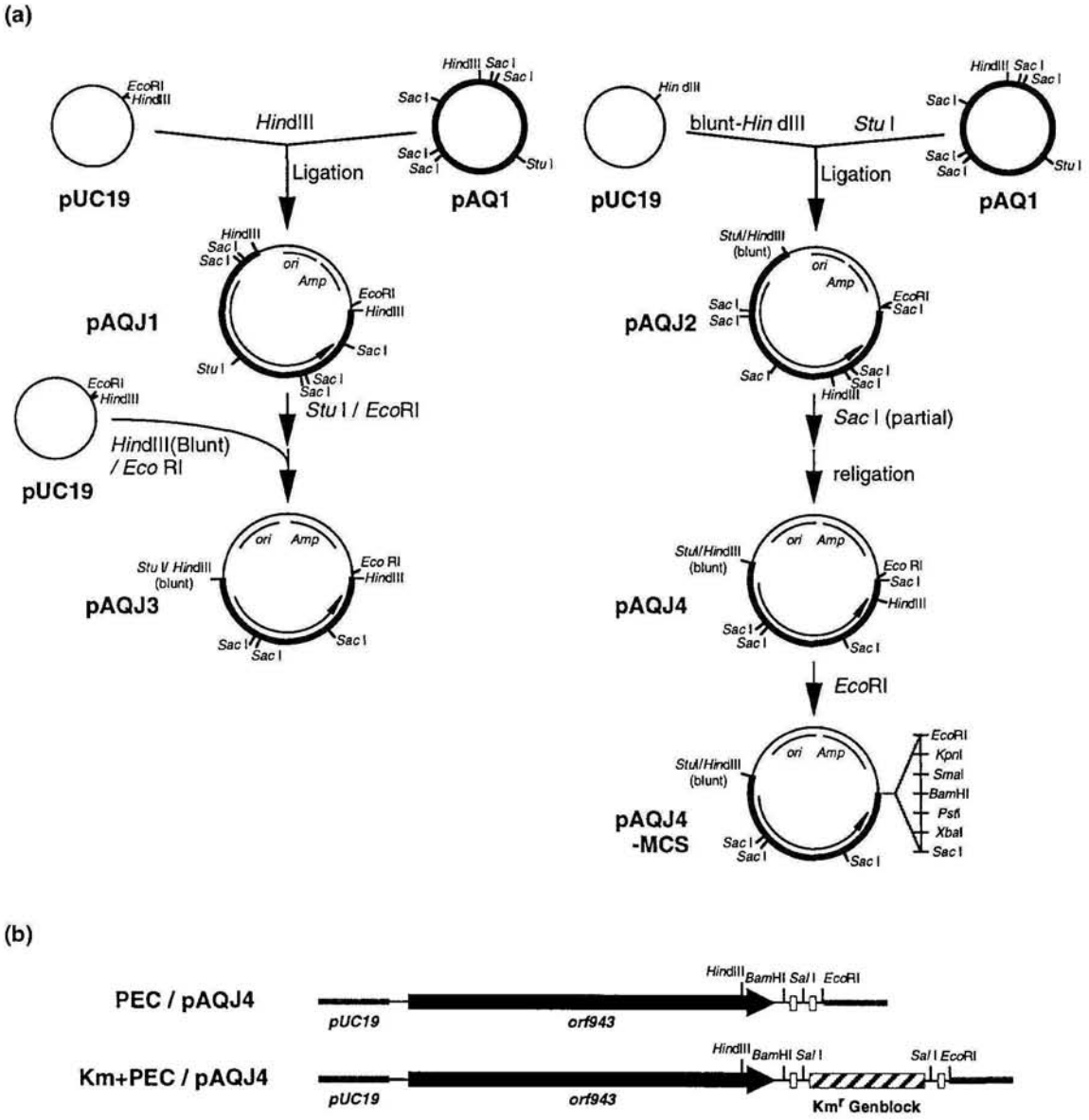


Figure 2. (a) Construction of a series of pAQJ vectors. The arrow indicates the direction of transcription of ORFs of pAQ1. Bold lines represent the pAQ1 sequences and the finer lines represent the pUC19 sequences. The vector pAQJ3 is a derivative of pAQJ1 from which approximately 2.5 kb DNA is removed. The vector pAQJ4 is a derivative of pAQJ2 and contains the full region of *orf943*. The pAQJ4-MCS contains six unique, available cloning sites as shown: *Eco*RI, *Kpn* I, *Sma* I, *Bam*HI, *Pst* I and *Xba* I. (b) Structure of a series of PEC vectors. A series of PEC vectors were constructed as described in Materials and Methods. The closed arrows indicate the direction of transcription of *orf943* of pAQJ4. Thin lines represent the pAQ1 DNA and the gray bold lines represent the pUC19 DNA. The open box shows a synthesized palindromic element.

2.4. Transformation and plasmid stability analysis

Transformation of *Synechococcus* sp. PCC7002 was performed by a slight modification of the previously described method[13] except that the concentration of DNA was modified to 0.4 nmol/ml. Transformed colonies appeared after about 4 days incubation. Plasmid DNA fractions from three independent transformants were prepared by the alkaline SDS method[11] and used to analyze the plasmid stability in cyanobacterial transformants by Southern hybridization.[11] An Amp^r probe (nt 1582–2274 in pUC19) was prepared from pUC19 by digestion with *Dra* I, and a pAQ1 probe was prepared from pAQ1 by digestion with *Hin*dIII.

The transformation mixtures were inoculated into fresh medium A with ampicillin (4 μg/ml) and autotrophically grown to the exponential growth phase under 1% CO_2 condition. In all cases, the plasmid isolated from each culture was amplified in *E. coli* JM109, and 20 in-

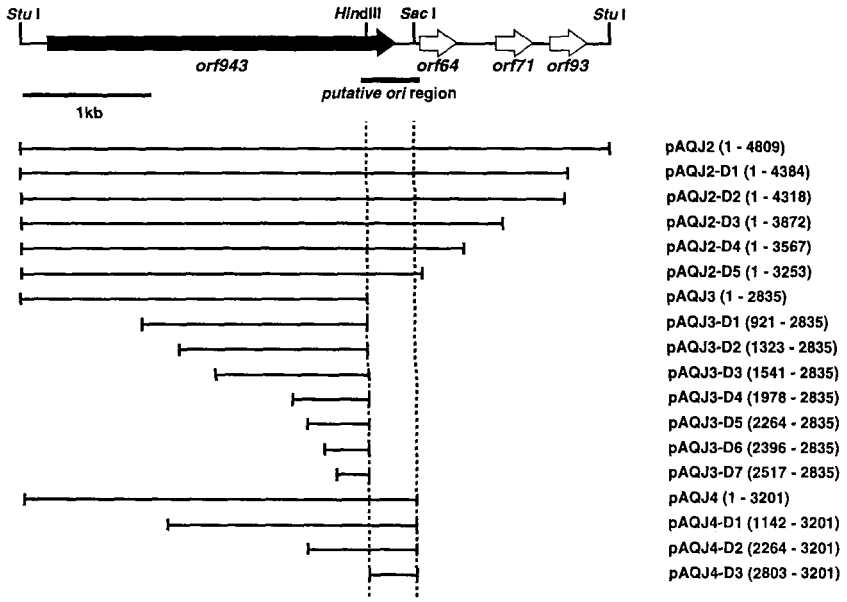


Figure 3. Alignment of the vectors used for the stability analysis. The extent and direction of transcription of *orf* identified are indicated by arrows: open (*orf93*, *orf71* and *orf64*) or closed (*orf943*). The putative replication origin (*ori*) region is marked with bold line. Regions (in bp), corresponding to the numbering in Fig. 1, are also shown with the name.

dependent *E. coli* transformants were screened by *Pvu* II restriction mapping and DNA sequence analysis, if needed.

The segregation of plasmids into daughter cells was analyzed as follows: cyanobacterial transformant harboring pAQJ4-MCS was grown to the exponential growth phase, inoculated (1:500) into fresh medium A without antibiotic selection and grown for (approximately ten generations). The culture was again diluted (1:500) and grown for 5 days until approximately 100 generations of unselected growth were reached. The cells were then diluted and plated on medium A with and without ampicillin (4 μg/ml). The plasmid isolated from the culture was analyzed by transformation back into *E. coli*, followed by *Pvu* II restriction mapping.

3. Results

3.1. Deletion analysis of the pAQ1-derived vectors

To determine the stabilities of plasmids in cyanobacterial transformants, two series of vectors pAQJ series and pAQJ-D series (Fig. 3), were constructed and transformed to the cyanobacterium. The constructs except pAQJ3-D6, pAQJ3-D7 and pAQJ4-D3 were found to be capable of transforming the cyanobacterium and conferring ampicillin resistance (Table 1). The vector pAQJ2 efficiently transformed into the cyanobacterium and the frequency of transformation was maintained even in the absence of three *orf* regions. All of these transformants contained intact plasmids after transformation back into *E. coli*; 20 independent *E. coli* transformants from each construct were tested, and in all cases plasmids showing the expected molecular sizes and *Pvu* II restriction pattern were recovered. In contrast, pAQJ3 (in which pAQ1 sequence downstream from *Hin*dIII site was deleted) transformed 20-fold lower than pAQJ2 did, and a decrease in frequency was also seen with pAQJ3 derivatives. None of them contained intact plasmids; plasmids from 20 independent *E. coli* transformants showed unexpected but similar molecular sizes and *Pvu* II restriction patterns. Thus, sequence analysis of resulting plasmids revealed that recombination(s) occurred between the introducing plasmid and a native pAQ1. Nevertheless, no sequence data obtained lacks any sequence or has an extra sequence at a point of cross-over. Only the fact that the recombination still occurred in pAQJ3-D5 but not in pAQJ3-D6 suggests that the recombination site(s) is (are) located between nt position 2264 and 2396 (Fig. 1).

The stability of each construct in cyanobacterial transformants was determined by Southern analysis. As shown in Fig. 4(a), pUC19 hybridizing sequences at positions expected for the constructs were observed in plasmid DNA fractions obtained from pAQJ1, pAQJ2, and pAQJ4 transformants, but not in those from pAQJ3 transformants. Since recombination between pAQJ3 and a native pAQ1 was observed after transformation back into *E. coli*, the lowest hybridizing material observed in the pAQJ3 transformant was a monomeric form of the resulting recombinant. It also shows that other hybridizing materials at the top of each lane represent closed- and open-circular forms of multimers of recombinants between pAQ1 and the introducing constructs and/or

Table 1. Efficiencies for introducing a series of pAQJ and pAQJ-D vectors and stabilities in the cyanobacterium.

vector	transformation frequency (transformant/μg DNA)	recombination (rec/total)
pAQJ1	2.9×10^6	0/20
pAQJ2	2.5×10^6	0/20
pAQJ2-D1	2.5×10^6	0/20
pAQJ2-D2	1.8×10^6	0/20
pAQJ2-D3	1.7×10^6	0/20
pAQJ2-D4	1.1×10^6	0/20
pAQJ2-D5	1.0×10^6	0/20
pAQJ3	1.3×10^5	20/20
pAQJ3-D1	1.6×10^4	20/20
pAQJ3-D2	2.9×10^3	20/20
pAQJ3-D3	3.1×10^3	20/20
pAQJ3-D4	3.0×10^3	20/20
pAQJ3-D5	2.1×10^3	20/20
pAQJ3-D6	$<2.0 \times 10^2$	ND
pAQJ3-D7	$<2.0 \times 10^2$	ND
pAQJ4	3.6×10^5	0/20
pAQJ4-D1	5.2×10^4	0/20
pAQJ4-D2	3.6×10^4	0/20
pAQJ4-D3	$<2.0 \times 10^2$	ND

Transformation of *Synechococcus* sp. PCC7002 was performed by a slight modification of the previous described method[13] except that the concentration of DNA was modified to 0.4 nmol/ml. Transformation efficiencies are expressed as cyanobacterial colonies per μg of plasmid DNA and are the mean values of 4–5 independent experiments selecting for resistance to ampicillin at a final concentration of 4 μg/ml. Analysis of plasmid stability was performed as follows: plasmid isolated from each transformant was amplified in *E. coli* JM109, and 20 independent *E. coli* transformants were screened by *Pvu* II restriction mapping. Recombination frequencies are given to the number of recombinants observed per number of plasmids screened. ND: not determined.

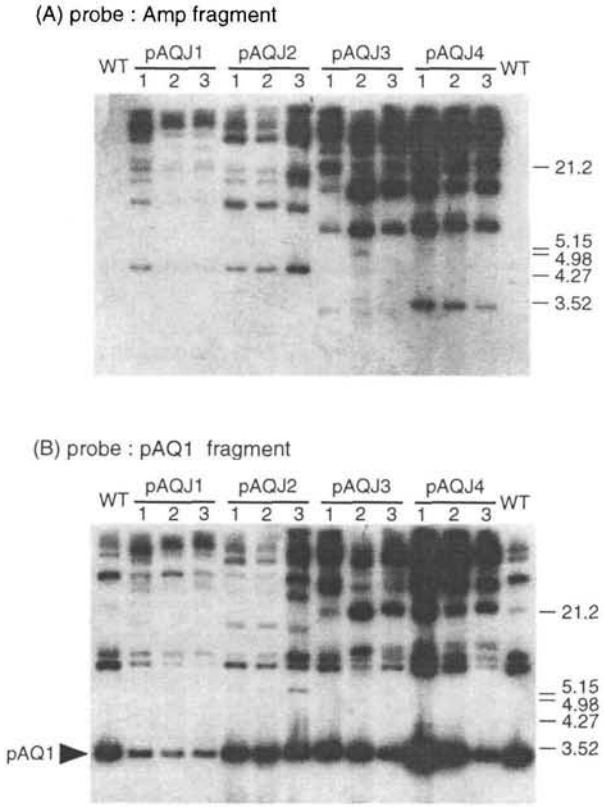


Figure 4. Southern blot analysis of plasmid DNA from cyanobacterial transformants. Plasmid DNA fractions from three independent cyanobacterial transformants were prepared by the alkaline SDS method[11] and used to analyze plasmid stability in cyanobacterial transformants. The ^{32}P radiolabeled Amp^r fragment and pAQ1 fragment were used as the hybridization probe. (A) The Amp^r fragment was used to analyze the stability of each construct. (B) The pAQ1 fragment was used to analyze the existence of a native pAQ1. The bars to the right show the position of size markers, the *Eco*RI and *Hin*dIII fragments of phage lambda DNA, whose sizes are indicated in kilobases. The arrow to the left show the position of pAQ1 observed from 1% agarose gel stained with ethidium bromide.

multimers of the introducing constructs. Furthermore, all transformants contained a native pAQ1, as shown in Fig. 4(b), indicating that plasmid incompatibility between pAQ1 and the introducing constructs did not cause the transformation. Taken together these results suggest that pAQJ3 and its derivatives have no ability to transform cyanobacterium and that this is probably because of the loss of the cyanobacterial *ori* region. However, once any recombinations between the constructs and a native pAQ1 occur, the resulting vector is stably maintained and the transformants were ampicillin resistant. Thus, the transformation efficiencies of pAQJ3 and its derivatives express the frequencies of recombination in the cyanobacterium—that is, the plasmid recombination in the cyanobacterium occurs with high frequency.

On the other hand, segregation analysis was performed to investigate the presence of stability-promoting functions on pAQJ4. pAQJ4 was shown to be stably maintained even after 100 generations of growth without antibiotic selection. The frequency of plasmid loss was about 0.5%, which means that approximately 1 in 400 cells produced a vector-free descendant per generation. Thus, it is concluded that pAQJ4 contains functions essential for replication and stability under this condition. It may be necessary to further investigate the recipient cell, pAQ1-free cyanobacterium, to determine whether or not *orf943* encodes a protein involved in replication and/or the maintenance of pAQ1 stability.

3.2. *Transformation of* Synechococcus *sp. PCC7002 with PEC vectors*

In order to investigate the function of the palindromic elements (G(G/A)CGATCGCC) on pAQ1, we constructed PEC and Km + PEC vectors and performed plasmid recombination analysis. Both se-

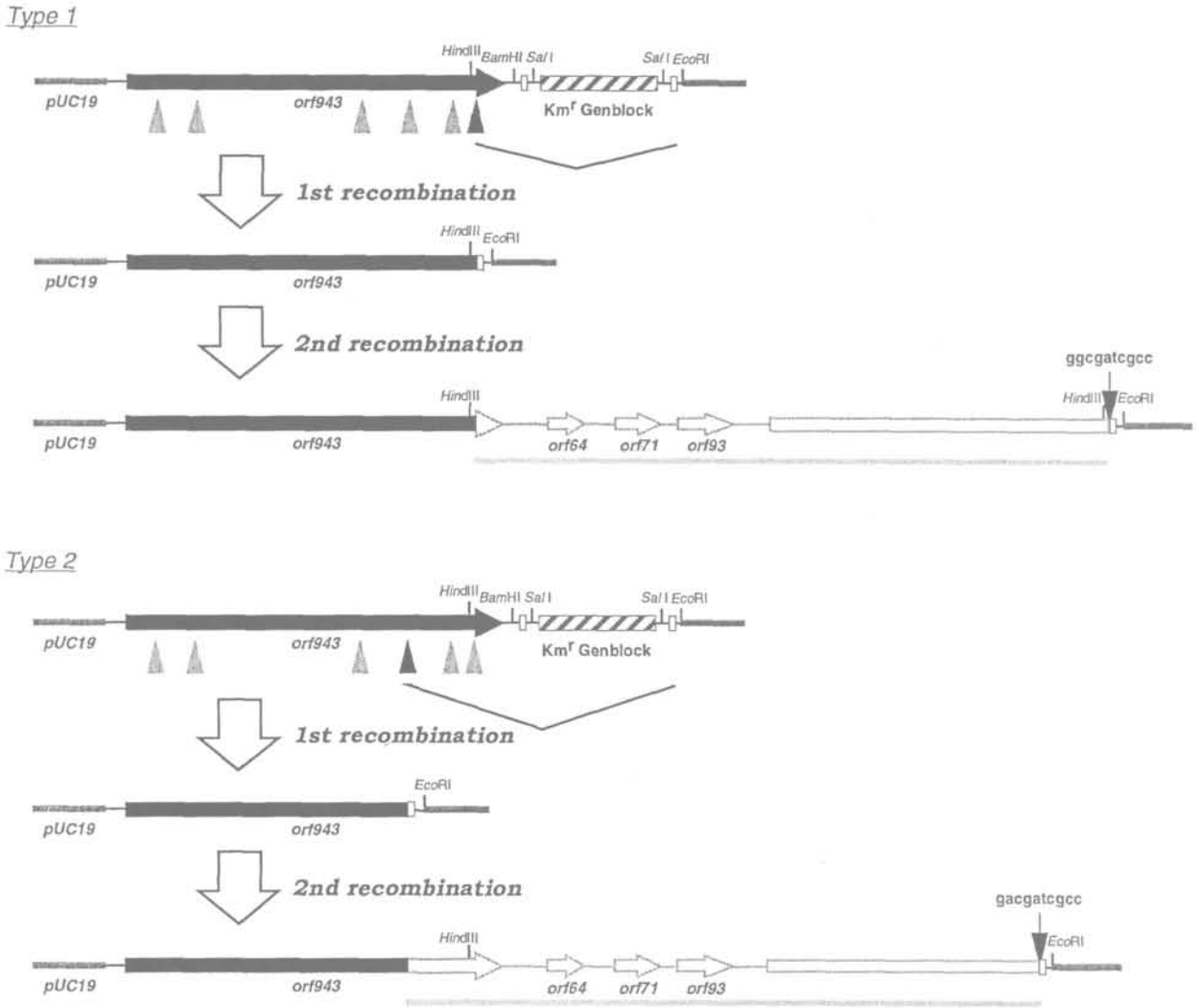


Figure 5. *Schematic illustration of plasmid recombination mechanism in* Km + PEC(WT:GG)/pAQJ4 *transformant.* The extent and direction of transcription of *orf* identified are shown as arrows. A family of 6 palindromic sequences G(G/A)CGATCGCC in *orf943* is indicated by triangles, and the recombination sites are shown by closed triangles. Open arrows represent the pAQ1 DNA recombined with the Km + PEC vector. In this model, the site-specific recombination in Km + PEC(WT:GG)/pAQJ4 transformant is a stepwise reaction; the first reaction is between the elements in the PEC vector and the second reaction is between the PEC vector and a native pAQ1 (Fig. 5). That is, in Km + PEC(WT:GG)/pAQJ4 transformant, the first recombination occurs between the synthesized element and either element in *orf943* region [nt 2365–2374 (type2) or nt 2988–2907 (type1)]. The resulting vector seems not to replicate in cyanobacteria because a putative *ori* region is excised by the deletion mechanism. The second recombination arises between the site recombined and either site in *orf943* region of a native pAQ1 and may be common in all constructs.

ries of vectors contain a set of palindromic elements, but the latter series contains an additional Km^r GenBlock spacer sequence (~1.5 kb) between the elements. Both PEC(AG) and Km + PEC(AG) vectors contain mutated (GGCAATCGCC) and normal (GGCGATCGCC) palindromic sequences, whereas PEC(AA) and Km + PEC(AA) vectors contain a set of mutated (GGCAATCGCC) palindromic sequences. These vectors are useful in probing the sequence specificity, and/or the requirement for symmetry in the palindromic sequence, and/or the spacing of the elements in the recombination events if observed.

All of the vectors constructed were capable of transforming the cyanobacterium as well as pAQJ4 did. However, recombination events were only observed in transformants harboring the Km + PEC(WT:GG) vector (Fig. 5). Half of plasmids (10 plasmids) isolated from 20 Amp^r cyanobacterial colonies were recombinants with native pAQ1. Sequence analysis of these recombinants showed that there were two recombination sites between the synthesized palindromic element and an element in *orf943* of a native pAQ1, and that the Km^r GenBlock was completely replaced by pAQ1; one was at nt position 2365–2674 and the other was at 2988–2907 (Fig. 5). Therefore, it is clear that both the sequence specificity and, in part, spacing of palindromic elements are needed

for a site-specific recombination of plasmids, since nucleotide substitution in the elements lose the recombination ability.

4. Discussion

To further our general understanding of the mechanism of plasmid recombination, we had two objectives in this study. The first is the characterization of pAQ1 derived shuttle-vectors and the second is the successful construction of a shuttle vector that is a suitable for understanding of plasmid recombination in the cyanobacterial cells. Several experiments aiming at the construction and characterization of shuttle vectors for cyanobacterial transformation have been carried out,[1–6] but the mechanisms of plasmid recombination are not well understood.

Recombination is the central phenomenon underlying genetic processes and the principal tool in genetic analysis, gene mapping and gene targeting. Since recombination in cyanobacterial cells occurs between DNA molecules with long stretches of homology, the key reaction is considered to be the pairing of arbitrary but homologous DNA molecules.[4,5] However, our results show that the mechanism of plasmid recombination is a site-specific recombination, not a homologous recombination. Since no recombinations were observed in transformant harboring PEC(WT:GG)/pAQJ4, the observed site-specific recombination in Km + PEC(WT:GG)/pAQJ4 transformant seems a stepwise reaction; the first is between the elements in the PEC vector and the second is between the PEC vector and a native pAQ1 (Fig. 5). That is, in Km + PEC(WT:GG)/pAQJ4 transformant, the first recombination occurs between the synthesized element and either element in the *orf943* region (nt 2365–2374 or nt 2988–2907) at a low frequency. The resulting vector seems not to replicate in cyanobacteria because a putative *ori* region is excised by the deletion mechanism. In fact, native forms of pAQJ3 and its derivatives (neither of them contain the putative *ori* region) were not found among the cyanobacterial transformants, indicating that the putative *ori* region is essential for plasmid replication and/or maintenance in cyanobacterium. The second reaction arises between the site recombined and either site in *orf943* region of a native pAQ1. In this model, each of two elements functions as recombination signal sequence, although the palindromic element is over-represented in pAQ1.[8] The latter mechanism would be also occurred in all constructs, since plasmid DNA from cyanobacterial transformants contain several hybridizing materials showing higher molecular size, which seems to be a multimer of recombinants between constructs and pAQ1 and/or multimers of constructs. Nevertheless, the only construct from which multimers were recovered was pAQJ3 and its derivatives after transformation back into *E. coli*, suggesting that plasmids showing higher molecular size can not replicate in *E. coli* and/or are poorly transformed in *E. coli*.

On the other hand, this element is quite similar to an octameric highly iterated palindrome (HIP1), GCGATCGC of *Synechococcus* sp. PCC6301.[14,15] As a result of long exposure to heavy metals for the cyanobacterium, a rearrangement occurred at the HIP1 site and the cyanobacterium was able to acquire heavy metal-resistance. This indicates that the HIP1 site functions as resolution site for site-specific recombination. Furthermore, the core sequence of HIP1, CGATCG, is over-represented in the accumulated cyanobacterial sequences from *Synechococcus* sp. PCC6301, PCC7002, PCC7942, *vulcanus* and *Synechocystis* sp. PCC6803; the average frequency of occurrence within the database are once every 562 nt, 785 nt, 479 nt, 1737 nt and 1731 nt, respectively (that for a 6-bp palindrome is $4,^6$ 4096).[16] Thus, the site-specific recombination mechanism based on the 10-bp palindromic element may be common in the cyanobacteria described above.

In summary, we describe a novel site-specific plasmid recombination mechanism in the unicellular cyanobacterium *Synechococcus* sp. PCC7002. We show that a palindromic element, in which the core sequence, GGC(G/A)ATCGCC, functions as resolution site for site-specific recombination and that this recombination needs sequence specificity, symmetry in the core sequence and, in part, the spacing of the elements. Furthermore, the very high frequency of occurrence of the element is seen not only in *Synechococcus* sp. PCC7002, but also in *Synechococcus* sp. PCC6301, *Synechococcus* sp. PCC7942, *vulcanus* and *Synechocystis* sp. PCC6803. This strongly suggests that the site-specific recombination mechanism based on a palindromic element would be common in cyanobacteria.

Acknowledgments: We wish to thank Dr. Masahiko Ikeuchi of the Univ. of Tokyo for his helpful discussions, and Mr. Masaaki Sugimoto and Mr. Tadanori Watanabe for their help in the preparation of this manuscript.

References

1. Kuhlemeier, C. J. and Van Arkel, G. A. 1987, Host-vector systems for gene cloning in cyanobacteria, *Methods Enzymol*, **153**, 199–215.
2. Scanlan, D. J., Bloye, S. A., Mann, N. H., Hodgson, D. A., and Carr, N. G. 1990 Construction of *lacZ* promoter probe vectors for use in *Synechococcus*: application to the identification of CO_2-regulated promoters, *Gene*, **90**, 43–49.
3. Van der Plas, J., Oosterhoff-Teertstra, R., Borrias, M., and Weisbeek, P. 1992, Identification of replication and stability functions in the complete nucleotide sequence of plasmid pUH24 from the cyanobacterium *Synechococcus* sp. PCC7942, *Mol. Microbiol*, **6**, 653–664.
4. Kuhlemeier, C. J., Hardon, E. M., Van Arkel, G. A., and Van de Vate. 1985, Self-cloning in the cyanobacterium

Anacystis nidulans R2, *Plasmid*, **14**, 200–208.
5. Gendel, S. M. 1987, Instability of Tn5 inserts in cyanobacterial cloning vectors, *J. Bacteriol*, **169**, 4426–4430.
6. Marraccini, P., Bulteau, S., Cassier-Chauvat, C., Mermet-Bouvier, P., and Chauvat, F. 1993, A conjugative plasmid vector for promoter analysis in several cyanobacteria of the genera *Synechococcus* and *Synechocystis*, *Plant Mol. Biol*, **23**, 905–909.
7. Robert, T. M. and Koths, K. E. 1976, The blue-green alga *Agmenellum quadruplicatum* contains covalently closed DNA circlesg, *Cell*, **9**, 551–557.
8. Akiyama, H., Kanai, S., Hirano, M., and Miyasaka, H. 1998, Nucleotide sequence of plasmid pAQ1 of cyanobacterium *Synechococcus* sp. PCC7002, *DNA Res.*, **5**, 127–129.
9. Scott, J. R. 1984, Regulation of plasmid replication. *Microbiol Rev*, **48**, 1–23.
10. Tabita, E. R., Stevens Jr., S. E., and Quijano, R. 1974, D-Ribulose 1,5-diphosphate carboxylase from blue-green algae, *Biochem. Biophys. Res. Commun.*, **61**, 45–52.
11. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1989, Molecular Cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
12. Henicoff, S. 1984, Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing, *Gene*, **28**, 351–359.
13. Buzby, J. S., Porter, R. D., and Stevens Jr., S. E. 1985, Expression of the *Escherichia coli lac*Z gene on a plasmid vector in a cyanobacterium, *Science*, **230**, 805–807.
14. Gupta, A., Morby, A. P., Turner, J. S., Whitton, B. A., and Robinson, N. J. 1993, Deletion within the metallothionein locus of cadmium-tolerant *Synechococcus* PCC6301 involving a highly iterated palindrome (HIP1), *Mol. Microbiol*, **7**, 189–195.
15 Huckel, J. W., Morby, A. P., Turner, J. W., and Robinson, N. J. 1993, Isolation of a prokaryotic metallothionein locus and analysis of transcriptional control by trace metal ions, *Mol. Microbiol*, **7**, 177–187.
16. Robinson, N. J., Robinson, P. J., Gupta, A., Bleasby, A. J., Whitton, B. A., and Morby, A. P. 1995, Singular over-representation of an octameric palindrome, HIP1, in DNA from many cyanobacteria, *Nucleic Acids Res.*, **23**, 729–735.