JOURNAL OF BACTERIOLOGY, June 1996, p. 3380–3383 Vol. 178, No. 11
0021-9193/96/$04.00+0

Analysis of Suppressor Mutations of *spoIVCA* Mutations: Occurrence of DNA Rearrangement in the Absence of a Site-Specific DNA Recombinase SpoIVCA in *Bacillus subtilis*

TSUTOMU SATO, KENJI HARADA, AND YASUO KOBAYASHI*

Department of Applied Biological Science, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan

Received 5 February 1996/Accepted 25 March 1996

The *spoIVCA* gene of *Bacillus subtilis* encodes a site-specific recombinase, which excises a 48-kb *skin* element from the chromosomal DNA by DNA rearrangement and creates a new composite gene, *sigK*, on the chromosome. From *spoIVCA* mutants, we have isolated Spo$^+$ revertants which have no *skin* element but have an intact *sigK* gene. This result suggests that the DNA rearrangement can occur in the absence of *spoIVCA*.

In a nutrient-deficient condition, *Bacillus subtilis* cells form an asymmetric septum to produce the small forespore and the much larger mother cell. During sporulation, DNA rearrangement occurs only in the mother cell by a site-specific recombinase, SpoIVCA (17), which excises a 48-kb *skin* element (20) from the chromosome (9) and creates a new composite gene, *sigK*, by the fusion of the *spoIIIC* and *spoIVCB* genes (4, 10, 19). The N-terminal region (approximately 150 amino acids) of SpoIVCA is similar to resolvase and invertase families (17). In addition, the entire amino acid sequence of the *spoIVCA* product is homologous to that of XisF (3), which excises a 55-kb element in *Anabaena* sp. strain PCC 7120 during heterocyst differentiation; to that of ORF469, an excision enzyme (11) of *Streptomyces parvulus* temperate phage R4; and to that of ORF3, an excision-integration enzyme (8) of *B. subtilis* temperate phage ϕ105. The *spoIVCA* gene governs developmentally regulated DNA rearrangement (17). Expression of *spoIVCA* is regulated by mother cell sigma factor σ^E and a regulatory protein, SpoIIID (7, 15), and SpoIVCA binds to the sequences (21-bp inverted repeat) of the flanking region of the *skin* element (12).

Isolation of suppressors of *spoIVCA* mutations. In an attempt to isolate genes whose products might interact with SpoIVCA and regulate the sporulation process, we first characterized *spoIVCA* mutants 801, 807, 816, 818, and 819 obtained from M. Yudkin (5) (Table 1). Since the mutation site of each *spoIVCA* mutation was unknown, we determined the mutation sites by PCR amplification and sequencing. The *spo-801*, *spo-807*, *spo-816*, *spo-818*, and *spo-819* mutations occurred in codons 314 (G→E), 421 (Q→ochre), 431 (Q→amber), 216 (Q→amber), and 120 (A→T) of the *spoIVCA* gene, respectively. These mutation sites are in good agreement with those indicated by reciprocal, three-factor mapping (5). The sporulation frequencies of these *spoIVCA* mutants ranged from 10^2 to 10^4 spores per ml (5). Twelve spontaneous Spo$^+$ revertants, sup1-1 (*spo-801 sup1-1*), sup1-3 (*spo-801 sup1-3*), sup1-5 (*spo-801 sup1-5*), sup7-1 (*spo-807 sup7-1*), sup7-4 (*spo-807 sup7-4*), sup16-1 (*spo-816 sup16-1*), sup16-4 (*spo-816 sup16-4*), sup16-5 (*spo-816 sup16-5*), sup18-1 (*spo-818 sup18-1*), sup19-1 (*spo-819 sup19-1*), sup19-2 (*spo-819 sup19-2*), and sup19-4 (*spo-819 sup19-4*), were isolated at a frequency of $<10^{-9}$ from these strains. The sporulation frequency of these Spo$^+$ revertants was restored to 10^8 spores per ml.

DNA rearrangement in the suppressor mutants. To see whether DNA rearrangement occurs in the revertants, Southern hybridization experiments were carried out with chromosomal DNA from vegetative cells and sporulating cells (T_6 cells; 6 h after the initiation of sporulation) grown at 37°C in Schaeffer's sporulation medium (18). The probe, a 3.6-kb *Eco*RI fragment carrying the *spoIVCA* and *spoIVCB* genes, gave a signal at 3.6 kb for vegetative DNAs and two new signals at 5.4 kb (corresponding to the excised *skin* element) and 2.8 kb (corresponding to the rearranged *sigK* gene) when the DNA rearrangement properly occurred (Fig. 1). Although all *spoIVCA* mutants were deficient in DNA rearrangement (17), nine *sup* mutants (sup1-1, -7-1, -7-4, -16-1, -16-4, -16-5, -19-1, -19-2, and -19-4) restored DNA rearrangement to the wild-type level. However, three other strains (sup1-3, -1-5, and -18-1) showed only a single signal, i.e., at 2.8 kb (Fig. 1B). This observation suggests that these three mutants have no *skin* element but have the rearranged *sigK* gene.

Detection of the *sigK* gene in the vegetative cells of *skin*-less mutants sup1-3, -1-5, and -18-1. To detect the *sigK* gene directly in vegetative cells of *sup* mutants sup1-3, -1-5, and -18-1, we performed PCR analysis (14) using two primers whose sequences are located in *spoIVCB* (primer 1; 5′-GCAGAGG ACTTAATCTCC-3′) and *spoIIIC* (primer 2; 5′-CGAAGA CGTGAAGAAGATAC-3′), respectively. As shown in Fig. 2, a 251-bp PCR product which corresponds to the size of a fragment containing the *spoIVCB-spoIIIC* joint region was detected in both vegetative and T_6 cells of the three mutants, whereas it was detected only in T_6 cells of the wild-type strain JH642. The nucleotide sequence of the 251-bp fragment obtained from these mutants was completely identical to that of JH642 (data not shown). These results strongly suggest that the mutants have a rearranged *sigK* gene in vegetative-phase cells.

Isolation of Spo$^+$ revertants from a *spoIVCA* null mutant. The results described above suggest that the *skin*-less mutants arose by *spoIVCA*-independent excision, since *spo-818* is a nonsense mutation in the N-terminal region of SpoIVCA. However, nonsense mutations can be slightly leaky; therefore, we constructed a *spoIVCA* null mutant, which was made by replacing the region between the primer 3 (5′-GTTCCTCG

* Corresponding author. Phone: 81-423-67-5706. Fax: 81-423-67-5715. Electronic mail address: ykobayas@cc.tuat.ac.jp.

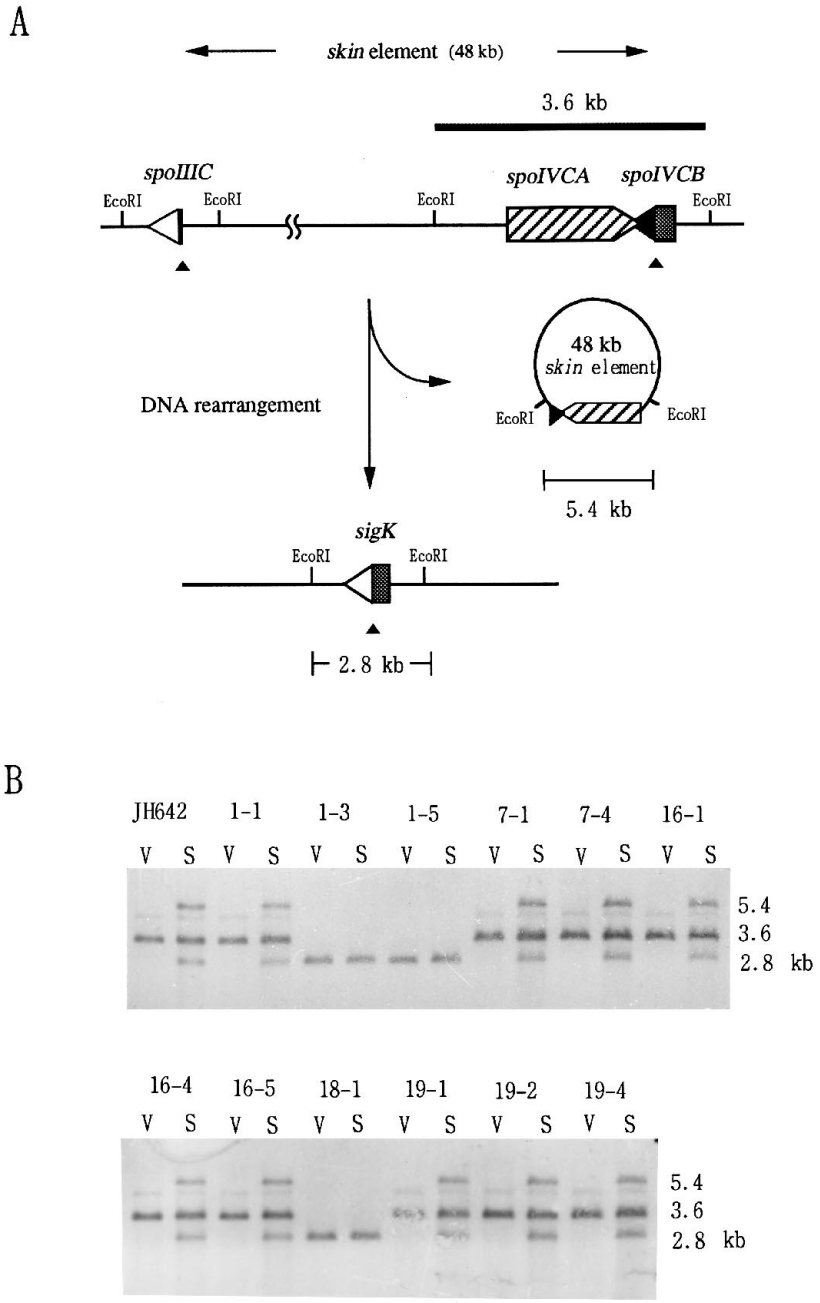

FIG. 1. DNA rearrangement in *sup* mutants. (A) *Eco*RI restriction map of the *spoIIIC-skin-spoIVCA-spoIVCB* region of the chromosome in vegetative cells (upper part) and T_6 mother cells (lower part). Thick bar, the region hybridized with the 3.6-kb *Eco*RI probe containing the *spoIVCA* and *spoIVCB* genes; closed triangle, DNA breakpoints. (B) Southern analysis of DNA isolated from the *sup* mutants. DNA samples were prepared from vegetative (V) and T_6 (S) cells of the wild type (strain JH642) and mutants. DNA samples were digested by *Eco*RI. The 3.6-kb *Eco*RI fragment was used as a probe.

GTCGATACCC) site (located 37 nucleotides downstream of the *spoIVCA* start codon) and the primer 4 (5′-ACTTGAG TATCAGCCCTCAC) site (located 866 nucleotides downstream of the *spoIVCA* start codon) with a *cat* cassette of ~1 kb (Fig. 3). We constructed pUCIVC, which consists of a 2.7-kb pUC18 plasmid and a 3.6-kb *Eco*RI fragment (17) containing *spoIVCA* (Fig. 3). This plasmid was used as a template for PCR amplification with primer 3 and primer 4, which are oriented to amplify a 5.5-kb fragment containing the 2.7-kb pUC18 and a part of *spoIVCA*. The resulting PCR product (5.5 kb in size) was treated with T4 DNA polymerase. An ~1-kb *Sma*I-*Hin*cII fragment carrying a *cat* cassette isolated from pCBB31 (21) was ligated with the PCR product (blunt end). The resulting plasmid, pUCIVCΔ, was linearized with *Eco*RI and was used to transform competent cells of strains 1012 ($RecA^+$) and 4309 ($RecA^-$) to chloramphenicol resistance to generate strains 4C12 (Δ*spoIVCA*::*cat*) and 4C09 (Δ*spoIVCA*::*cat recA4*), respectively. The sporulation of *spoIVCA* null mutants was completely blocked (data not shown). We could isolate two Spo^+ revertants (sup-120 and sup-471) from 1,000 24-h cultures (1 ml each) of 4C12 (this frequency is lower than that obtained with *spoIVCA* point mutants); however, no Spo^+ revertant was

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Source or reference
JH642	*trpC2 pheA1*	Laboratory stock
1012	*leuA8 metB5 nonB1*	2
4309[a]	*metB5 nonB1 recA4*	2
801	*lys-1 purB6 spo-801*	5
807	*lys-1 purB6 spo-807*	5
816	*lys-1 purB6 spo-816*	5
818	*lys-1 purB6 spo-818*	5
819	*lys-1 purB6 spo-819*	5

[a] Strain 4309 was derived from strain 1012 by congression.

obtained from 4C09. PCR analysis of these two Spo$^+$ revertants with primer 1 and primer 2 showed the presence of the 251-bp fragment corresponding to the size of the *spoIVCB-spoIIIC* joint region in the vegetative chromosomes of the two strains (Fig. 2). This result indicated that the revertants have the *sigK* gene in their vegetative cells and have no *skin* element and that DNA rearrangement can occur in the complete absence of *spoIVCA*.

Mutation sites of *spoIVCA* in the *sup* mutants. We mapped the suppressing mutations in nine other Spo$^+$ revertants of *spoIVCA* mutants. For mapping, we inserted a *cat* gene in the *Eco*RV site located 695 nucleotides upstream of the *spoIVCA* start codon (a nonessential region for vegetative growth or sporulation) in the chromosomes of revertant *spoIVCA* mutants. These DNAs were then used to transform the respective *sup* mutants to Cm^r. Transformants of eight strains (sup1-1, -7-1, -7-4, -16-4, -16-5, -19-1, -19-2, and -19-4) showed a Spo$^-$ Cm^r phenotype, suggesting that these strains have a second mutation in the *spoIVCA* gene (intragenic suppressor). On the other hand, strain sup16-1 showed a Spo$^+$ Cm^r phenotype, indicating that this strain has an intergenic suppressor. We cloned and sequenced the *sup16-1* gene and found that the *sup16-1* mutation is a change in the anticodon of tRNA^{Lys} (UUU to UUA) in the *rrnB* operon (13). This ochre suppressor is identical to the *sup-3* gene reported by Garrity and Zahler (6).

Concluding remarks. We have obtained *skin*-less strains from *spoIVCA* mutants. The *skin*-less mutants presumably arose by *spoIVCA*-independent excision. We have preliminary evidence that *recA*-dependent homologous recombination between the ends (5-bp direct repeat) (19) of the *skin* element is responsible for this excision, since the *skin*-less mutant could not be obtained from *recA* mutant. However, the frequency of generating *skin*-less strains from a *spoIVCA* null mutant was much lower than that from the *spoIVCA* point mutants. This result suggests the possibility that SpoIVCA-dependent recombination also occurs in vegetative cells or forespores of the wild-type strain at low frequency.

Thus far, several *skin*-less *Bacillus* species are known. For instance, the *Bacillus thuringiensis* sigma factor σ^{28} shows 85% similarity to σ^K of *B. subtilis*; however, no *skin*-like sequence

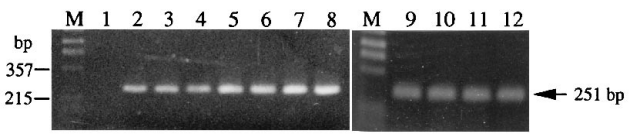

FIG. 2. PCR amplification of the *sigK* gene. DNA samples were extracted from vegetative cells (lanes 1, 3, 5, 7, 9, and 11) and T_6 cells (lanes 2, 4, 6, 8, 10, and 12). M, M13mp18 *Hpa*II digest. Lanes: 1 and 2, JH642; 3 and 4, sup1-3; 5 and 6, sup1-5; 7 and 8, sup18-1; 9 and 10, sup-120; 11 and 12, sup-471.

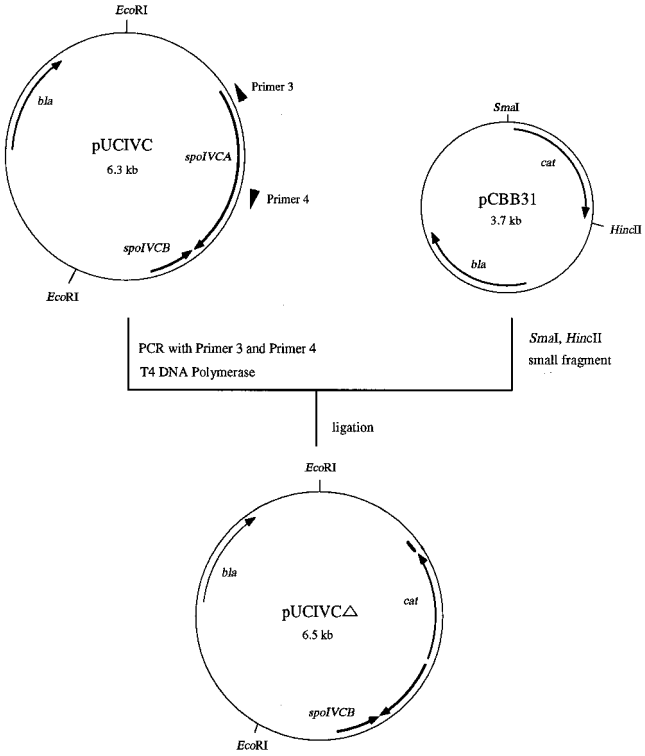

FIG. 3. Schematic outline of construction of plasmid pUCIVCΔ. The procedures used are described in the text.

has been detected in the vegetative DNA of *B. thuringiensis* (1). Similarly, no *skin* element has been detected in some *B. subtilis* strains isolated from natural environments (16). These *skin*-less Spo$^+$ strains may have arisen by homologous recombination between the ends of the *skin* element in vegetative cells or forespores during the course of evolution. Our discovery of an arsenate-inducible arsenic resistance operon within the *skin* element (20; data not shown) may suggest a reason for the maintenance of the *skin* element in commonly used *B. subtilis* strains.

We thank M. Itaya for helpful discussions.

This work was supported in part by a grant for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. **Adams, L. F., K. L. Brown, and H. R. Whiteley.** 1991. Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. J. Bacteriol. **173:**3846–3854.
2. **Anaguchi, H., S. Fukui, H. Shimotsu, F. Kawamura, H. Saito, and Y. Kobayashi.** 1984. Cloning of sporulation gene *spoIIC* in *Bacillus subtilis*. J. Gen. Microbiol. **130:**757–760.
3. **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.
4. **Errington, J., S. Rong, M. S. Rosenkrantz, and A. L. Sonenshein.** 1988. Transcriptional regulation and structure of the *Bacillus subtilis* sporulation locus *spoIIIC*. J. Bacteriol. **170:**1162–1167.
5. **Farquhar, R., and M. D. Yudkin.** 1988. Phenotypic and genetic characterization of mutations in the *spoIVC* locus of *Bacillus subtilis*. J. Gen. Microbiol. **134:**9–17.
6. **Garrity, D. B., and S. A. Zahler.** 1993. The *Bacillus subtilis* ochre suppressor *sup-3* is located in an operon of seven tRNA genes. J. Bacteriol. **175:**6512–6517.
7. **Halberg, R., and L. Kroos.** 1994. Sporulation regulatory protein SpoIIID from *Bacillus subtilis* activates and represses transcription by both mother-

cell-specific forms of RNA polymerase. J. Mol. Biol. **243:**425–436.
8. **Kobayashi, K., T. Sato, and Y. Kobayashi.** Unpublished results.
9. **Kunkel, B., R. Losick, and P. Stragier.** 1990. The *Bacillus subtilis* gene for the developmental transcription factor σ^K is generated by excision of a dispensable DNA element containing a sporulation recombinase gene. Genes Dev. **4:**525–535.
10. **Kunkel, B., K. Sandman, S. Panzer, P. Youngman, and R. Losick.** 1988. The promoter for a sporulation gene in the *spoIVC* locus of *Bacillus subtilis* and its use in studies of temporal and spatial control of gene expression. J. Bacteriol. **170:**3513–3522.
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 prophage genome from the chromosome of a lysogen. J. Gen. Appl. Microbiol. **41:**53–61.
12. **Popham, D. L., and P. Stragier.** 1992. Binding of the *Bacillus subtilis spoIVCA* product to recombination sites of the element interrupting the σ^K-encoding gene. Proc. Natl. Acad. Sci. USA **85:**5991–5995.
13. **Rudner, R., A. Chevrestt, S. R. Buchholz, B. Studamire, A.-M. White, and E. D. Jarvis.** 1993. Two tRNA gene clusters associated with rRNA operons *rrnD* and *rrnE* in *Bacillus subtilis*. J. Bacteriol. **175:**503–509.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Sato, T., K. Harada, Y. Ohta, and Y. Kobayashi.** 1994. Expression of the *Bacillus subtilis spoIVCA* gene, which encodes a site-specific recombinase, depends on the *spoIIGB* product. J. Bacteriol. **176:**935–937.
16. **Sato, T., P. Lisdiyanti, and Y. Kobayashi.** Unpublished results.
17. **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.
18. **Schaeffer, P., J. Millet, and J. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
19. **Stragier, P., B. Kunkel, L. Kroos, and R. Losick.** 1989. Chromosomal rearrangement generating a composite gene for a developmental transcription factor. Science **243:**507–512.
20. **Takemaru, K., M. Mizuno, T. Sato, M. Takeuchi, and Y. Kobayashi.** 1995. Complete nucleotide sequence of a *skin* element excised by DNA rearrangement during sporulation in *Bacillus subtilis*. Microbiology **141:**323–327.
21. **Yamada, K.** 1989. Ph.D. thesis. Hiroshima University, Hiroshima, Japan.