JOURNAL OF BACTERIOLOGY, Oct. 1988, p. 4791–4797
0021-9193/88/104791-07$02.00/0

Vol. 170, No. 10

Cloning and Characterization of a *Bacillus subtilis* Transcription Unit Involved in ATP-Dependent DNase Synthesis

JAN KOOISTRA,* BEN VOSMAN, AND GERARD VENEMA

Department of Genetics, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Received 29 February 1988/Accepted 15 July 1988

By insertional mutagenesis, 36 transformation-deficient, mitomycin C-sensitive *Bacillus subtilis* mutants were isolated, 16 of which were ATP-dependent DNase (ADD) deficient. PBS1 transduction showed that the mutations were closely linked to the *metD* marker and weakly linked to the *glyB* marker. With the aid of one of the mutants, a transcription unit involved in ADD synthesis was cloned. The chromosomal location of the transcription unit was established at the restriction site level by determining the presence or absence of ADD in transformants of wild-type cells obtained with various DNA fragments inserted in pUC derivatives. The transcription unit complemented a mutant in which the *add* transcription unit had been deleted.

The presence of an ATP-dependent DNase (ADD) has been observed in many organisms (for a review, see reference 43). In *Escherichia coli*, three genes (*recB*, *recC*, and *recD*) are involved in the synthesis of this enzyme. *recB*, *recC*, and *recD* encode three proteins with molecular weights of 135,000, 125,000, and 58,000, respectively (1, 21). *E. coli recB recC* mutants, lacking ADD activity, exhibit reduced homologous recombination in conjugation and generalized transduction (7) and are impaired in their ability to repair DNA damage (7).

The RecBCD enzyme has various biochemical activities: ATP-dependent double-stranded DNA exonuclease activity (18, 36), single-stranded DNA exo- and endonuclease activity (17), ATP-dependent DNA unwinding activity (39, 42), and ATP-dependent ATPase activity (18).

With respect to the composition of the *Bacillus subtilis* ADD enzyme, the available data do not agree. According to Doly and Anagnostopoulos (10), the enzyme consists of five subunits with molecular weights of 81,000, 70,000, 62,000, 52,000, and 42,000. However, Chestukhin et al. (6) have reported that the enzyme is composed of only two subunits with molecular weights of 140,000 and 155,000. The *B. subtilis* ADD enzyme is endowed with DNA-dependent ATPase activity (10) and double-stranded DNA exonuclease activity but not with single-stranded DNA nuclease activity (11). The ADD-deficient *recE5* mutant of *B. subtilis* (12) shows reduced transformation with chromosomal DNA and is poorly transducible (12). Although Chaudhury and Smith (5) have suggested that the DNA helicase activity of the RecB RecC subunits of the enzyme may play a role in recombination in *E. coli*, the reasons why ADD deficiency results in recombination deficiency and inability to repair DNA damage are largely unknown.

In order to study the role of the ADD enzyme in recombination in *B. subtilis*, we attempted to clone the genes involved in ADD synthesis with insertion of the vector pHV60 (32, 35), which allows both the production of mutants and the cloning of *B. subtilis* genes.

The present paper describes the isolation of ADD-deficient mutants of *B. subtilis*, the cloning of one of the transcription units involved in ADD synthesis, and the localization of the transcription unit on the chromosome.

* Corresponding author.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used are shown in Table 1.

Media. *B. subtilis* minimal growth medium consisted of Spizizen minimal salts (41), supplemented with glucose (0.5%) and casein hydrolysate (0.02%; Difco Laboratories, Detroit, Mich.). Amino acids, nucleotides (20 μg/ml each), or vitamins (0.4 μg/ml each) were added if required. Minimal agar consisted of minimal salts supplemented with 0.5% glucose, the required growth factors, and 1.5% agar. TY medium and TY agar were prepared as described by Biswal et al. (2). Antibiotic medium 3 was prepared as recommended by the manufacturer (Difco). Antibiotic medium 2 was prepared as described by Smith et al. (40). Trypticase agar was prepared by the method of Frischauf et al. (16).

Chemicals and enzymes. The chemicals used were of analytical grade and were obtained from E. Merck AG, (Darmstadt, Federal Republic of Germany) or BDH (Poole, England). Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were used as recommended by the manufacturer (Boehringer GmbH, Mannheim, Federal Republic of Germany).

Isolation of DNA. The *B. subtilis* chromosomal DNA and plasmid DNA were isolated as described by Venema et al. (44) and by the method of Ish-Horowicz and Burke (26), respectively. Radioactive DNA was isolated from strain 2G8, grown in minimal medium supplemented with 20 μg of tyrosine per ml, 2 μg of thymidine per ml, and [*methyl*-^{3}H]thymidine (10 μCi; specific activity, 20.7 mCi/mg; Amersham International plc, Amersham, United Kingdom). The specific activity of the isolated radioactive DNA was 1.6×10^5 cpm/μg of DNA. DNA from bacteriophage λEMBL4 was isolated according to the method described by Frischauf et al. (16).

Competence and transformation. *B. subtilis* cells were grown until they were competent, as described by Bron and Venema (3). After exposure of the cells to chromosomal 0G1 DNA (2 μg/ml) for 45 min, *trp*$^+$ transformants were selected on supplemented minimal agar plates. In transformations with plasmid DNA, the cells were exposed to DNA for 30 min, and the cultures were then diluted twice with TY medium, incubated for 1 h at 37°C, and plated on TY agar plates containing chloramphenicol (Cm) (5 μg/ml), kanamycin (20 μg/ml), or erythromycin (1 μg/ml). When erythromycin-resistant (Em^r) transformants were selected, the cultures

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype, phenotype, or plasmid marker	Source or reference
B. subtilis		
0G1	Prototrophic	3
8G5	*trpC2 tyr-1 his ade met rib ura nic*	3
2G8	*tyr-1 thy*	Laboratory collection
1A84	*glyB metD*	*Bacillus* Genetic Stock Center, Ohio State University, Columbus
QB934	*tre-12 metC3 glyB133 trpC2*	8
E1, E2, E6, E10, E16 E34, M5, M6, M11, M13	ADD-deficient mutants from 8G5	This paper
7G224	*trpC2 try-1 ade met rib ura nic recE4*	9
recH342		37
GSY2261 (*add*$^+$)	*hisH2 metB5*	C. Anagnostopoulos (personal communication)
GSY2258 (*add-5*), GSY2270 (*add-71*), GSY2266 (*add-72*)	*hisH2 metB5*; ADD deficient	12; C. Anagnostopoulos (personal communication)
E. coli		
JM83	*ara* Δ(*lac-proAB*) *rpsL* ϕ80 *lacZ* M15	45
BHB2600	*803 supE supF* r_K^- m_K^+ *met*	23
NM539	*supF hsdR* (P2 *cox-3*)	16
Plasmids		
pUB110	Km^r	19
pUC9, pUC19	Ap^r	3, 45
pGV1	Km^r	47
pHV60	Cm^r	32
pGV42, pGV49, pGV54, pGV55, pGV77, pGV133	Km^r	This paper
pKV42, pKV49	Cm^r	This paper
pC194	Cm^r	24
pE194	Em^r	25

were diluted twice with TY medium containing 0.05 μg of erythromycin per ml, incubated for 1 h at 37°C, and then plated.

E. coli was made competent and transformed by the method of Mandel and Higa (29).

Binding and entry of transforming DNA. Binding and entry of transforming DNA were determined as described by Mulder and Venema (33).

Cell survival following UV-irradiation. Cultures were grown in supplemented minimal medium to an A_{450} of 1.0 (approximately 10^8 cells per ml). The cells were washed twice with minimal medium containing 0.5% glucose and resuspended in the original volume of this medium. Samples (1 ml) were irradiated with a low-pressure mercury lamp (Hanovia, 256 nm, approximately 2 J/m^2/s) for various periods of time, and then cells were plated on TY agar plates.

Insertional mutagenesis. Insertional mutagenesis of *B. subtilis* with the vector pHV60 (32), was carried out essentially as described by Niaudet et al. (35).

Selection of transformation-deficient, MC-sensitive mutants. The Cm-resistant (Cm^r) transformants of *B. subtilis* obtained by insertion of pHV60 into the chromosome were first tested for transformation deficiency, as described by Smith et al. (40), and then for sensitivity to mitomycin C (MC) by transferring the Cm^r transformants to TY agar plates containing Cm (5 μg/ml) and MC (50 ng/ml). Transformants which failed to grow at an MC concentration of 50 ng/ml were considered to be MC sensitive, because wild-type cells were still able to grow on plates with 70 ng/ml.

Transduction and mapping. PBS1 transductions of strains 1A84 and QB934 were performed as described by Hoch et al. (22). *GlyB*$^+$, *metC*$^+$, and *metD*$^+$ transductants, obtained with PBS1 lysates of the mutants, were selected on minimal agar plates, supplemented with glycine (20 μg/ml) and methionine (20 μg/ml), respectively. To determine the linkage between the *metC*, *metD*, or *glyB* marker and the Cm^r marker of the integrated plasmid pHV60, the *metC*$^+$, *metD*$^+$, and *glyB*$^+$ transductants were transferred from the minimal agar plates to TY agar plates containing 5 μg of Cm per ml. After incubation overnight at 37°C, the numbers of *metC*$^+$, *metD*$^+$, and *glyB*$^+$ transductants that were Cm^r were determined. The map distances between the *metD* and the Cm^r marker and between the *glyB* and the Cm^r marker were expressed as follows: (1 − cotransfer index) × 100, which was calculated as described by Nester and Lederberg (34).

Assay of ADD activity. Cultures (5 ml) of *B. subtilis* grown in TY broth to an A_{600} of 1.0 were washed twice with 5 ml of an 0.05 M Tris hydrochloride buffer (pH 8.0), containing 0.1 mM EDTA and 0.1 mM dithiothreitol and the cells were resuspended in 0.5 ml of the same buffer and lysed by the addition of lysozyme (0.5 mg/ml) until lysis was complete (usually 15 min at 37°C). Samples (0.1 ml) of the lysate were added to 0.4 ml of a reaction mixture consisting of 0.1 M glycine-NaOH buffer (pH 9.2), 2.5 mM 2-mercaptoethanol, 0.05 M $MgCl_2$, 0.4 mg of bovine serum albumin per ml, and 75 μM ATP. Subsequently, 10 μl of a ^{3}H-labeled DNA solution (40 μg/ml; specific activity, 1.6×10^5 cpm/μg) was added, and after incubation for 30 min at 40°C, the reaction was stopped by chilling on ice and the addition of 0.2 ml of calf thymus DNA solution (2 mg/ml) and 0.15 ml of trichloroacetic acid (50% [wt/vol]). After 30 min at 0°C, the mixtures were centrifuged in an Eppendorf centrifuge for 10 min and 0.6 ml of the supernatant was added to 5 ml of Hydroluma scintillation fluid. Radioactivity was counted for 5 min.

Determination of amplification of the Cm^r marker. To determine whether the Cm^r marker in the mutants was amplifiable, the same method was used as described by Vosman et al. (46).

Isolation of fragments with the Em^r and the Cm^r marker from pE194 and pC194, respectively. A *Taq*I fragment of plasmid pE194 was inserted into the *Acc*I site of plasmid pUC7. From the resulting plasmid, an *Eco*RI fragment containing the Em^r marker was isolated.

From plasmid pC194, a fragment containing the Cm^r marker was isolated by partial digestion with *Dra*I. This fragment was inserted into a blunt-ended *Pst*I site of pUC7. From the resulting plasmid, a *Hin*dII fragment containing the Cm^r marker was isolated.

Construction of a *B. subtilis* genome bank in phage λEMBL4. The construction of this bank, consisting of 12- to 17-kilobase (kb) *Eco*RI fragments of *B. subtilis* DNA in phage λEMBL4, has been described previously (47).

Screening of the *B. subtilis* genome bank. To screen the *B. subtilis* genome bank, pKV42 was used as a probe. This plasmid was nick translated with [α-^{32}P]dCTP by the method of Rigby et al. (38). To isolate phages hybridizing with this probe, recombinant λEMBL4 was plated on Trypticase agar plates with *E. coli* NM539 as the indicator. The plaques, approximately 2,000 per plate, were transferred to a Gene ScreenPlus membrane (Dupont, NEN Research Products, Dreieich, Federal Republic of Germany), as described by Maniatis et al. (30), and then hybridized against the probe by the method recommended by the manufacturer. Five plaques hybridized with plasmid pK42. The purity of these phages was tested by repeated hybridization with pKV42.

RESULTS

Isolation of transformation-deficient, MC-sensitive mutants. Insertional mutagenesis was effected by transformation of an 8G5 strain of *B. subtilis* with plasmid pHV60, either containing *Eco*RI or *Mbo*I DNA fragments of wild-type (0G1) DNA, which had been inserted into the *Eco*RI and *Bam*HI site of the plasmid, respectively (32, 35). Plasmid pHV60 replicates in *E. coli*, but not in *B. subtilis*, and carries a Cm^r marker, which is expressed in both bacterial species (32). In transformation of the wild-type strain of *B. subtilis*, Cm^r transformants will be formed only if the vector has been integrated into the chromosome.

To select for recombination-deficient mutants, the Cm^r transformants (resistant to 5 μg of Cm per ml) were first tested for their transformability. Transformants impaired in transformation were further tested for their sensitivity to MC.

Of 9,000 Cm^r transformants obtained with the vector containing *Mbo*I fragments, 21 were impaired in transformation and were sensitive to MC. Of the 2,700 Cm^r transformants obtained with the vector containing *Eco*RI fragments, 15 were both transformation deficient and sensitive to MC.

Assay for ADD activity. The transformation-deficient, MC-sensitive mutants were assayed for ADD activity. Relatively high numbers of the mutants appeared to be deficient in ADD activity. Of the 21 mutants obtained with pHV60 and containing *Mbo*I fragments, 4 mutants (M5, M6, M11, and M13) appeared to be deficient in ADD activity; of the 15 mutants obtained with pHV60 and containing *Eco*RI fragments, 6 mutants (E1, E2, E6, E10, E16, and E34) appeared to be deficient in ADD activity (Table 2). Table 2 also shows that *recH342* (37), carrying a supposed *add* mutation, is not deficient in ADD activity. This finding is in agreement with that of C. Anagnostopoulos (personal communication), but in contrast with a previous report, in which it was claimed that this mutant was deficient in ADD activity (6, 37). The ADD-deficient mutants obtained by insertional mutagenesis were as deficient in ADD activity as the *add-5* mutant (*recE5* in reference 12) and the *add-71* and *add-72* mutants.

TABLE 2. ADD activity and mapping of the Cm^r marker in *add*-deficient mutants

Strain	Acid-soluble products (cpm) of [^{3}H]DNA formed		Map distance between the Cm^r marker of pHV60 and the following marker:	
	With ATP	Without ATP	*metD*	*glyB*
8G5	6,499	924		
E1	742	1,049	17	ND[a]
E2	668	663	24	ND
E6	1,168	1,187	25	73
E10	534	525	25	ND
E16	510	535	–[b]	80
E34	685	691	19	95
M5	1,216	1,521	–	62
M6	1,542	1,558	20	90
M11	1,050	858	23	ND
M13	1,046	811	23	ND
GSY2261 (*add*$^+$)	2,491	1,029		
GSY2258 (*add-5*)	1,179	1,125		
GSY2266 (*add-72*)	629	708		
GSY2270 (*add-71*)	686	940		
recH342	10,089	658		

[a] ND, Not determined.
[b] –, No *metD*$^+$ transductants found.

Mapping of *add* mutants. The *add* mutations were mapped with the use of the Cm^r marker of pHV60, which, through integration, had either interrupted or deleted the genes encoding ADD subunits (interruption occurs when pHV60 carries one chromosomal DNA fragment; a deletion is formed if the vector carries two nonadjacent chromosomal DNA fragments [35]).

The results of mapping of the Cm^r marker by PBS1 transduction are also shown in Table 2. The data show that, as far as could be tested, in all *add* mutants the Cm^r marker was linked closely to the *metD* marker and linked only weakly to the *glyB* marker. None of the *metC*$^+$ transductants were Cm^r, indicating that the marker was not linked to *metC*. Since according to Henner and Hoch (20), the map positions of the *glyB*, *metD*, and *metC* markers are 75, 95, and 115, respectively, the *add* mutations should be located between the *metD* and *glyB* marker.

To test whether in the *add* mutants the vector had been integrated by replacement recombination or by a Campbell-like mechanism, use was made of the finding of Janniere et al. (27) that in *B. subtilis*, only DNA structures integrated by a Campbell-like mechanism are amplifiable. Since none of the mutants was able to grow in the presence of increased amounts of Cm, apparently no amplification had occurred, suggesting that in all mutants the vector had been integrated by replacement recombination, resulting in the formation of deletions. This was supported by the observation that in transduction of a *metD* mutant strain with PBS1 lysates of the mutants M5 and E16, no *metD*$^+$ transductants were obtained. Apparently, the chromosomal region deleted in these mutants overlapped the *metD* marker.

Transformability and viability of the *add* mutants. To determine the extent of transformation deficiency in the *add* mutants, the cells were subjected to the standard competence regimen (3) and transformed with both chromosomal DNA from strain 0G1 and plasmid pUB110 DNA. No

TABLE 3. Transformability of *add* mutants

Strain[a]	Total no of viable cells/ml	No. of transformants/ml		Transformation frequency[b]	
		With chromosomal DNA (*trp*$^+$)	With plasmid pUB110 (Km^r)	With chromosomal DNA	With plasmid DNA
8G5	3.1×10^8	3.4×10^6	2.5×10^4	1.1×10^{-2}	8.0×10^{-5}
E1	1.4×10^7	3.6×10^3	7.2×10^2	2.6×10^{-4}	5.1×10^{-5}
E2	1.5×10^7	4.9×10^3	6.4×10^2	3.3×10^{-4}	4.3×10^{-5}
E6	2.7×10^7	0	0	0	0
E10	3.1×10^7	5.4×10^3	1.9×10^3	1.7×10^{-4}	6.0×10^{-5}
E16	1.3×10^7	0	0	0	0
E34	2.6×10^7	0	0	0	0
M5	2.7×10^7	0	0	0	0
M6	3.2×10^7	6.4×10^3	1.6×10^3	2.0×10^{-4}	5.0×10^{-5}
M11	1.6×10^7	0	0	0	0
M13	1.2×10^7	2.1×10^3	4.5×10^2	1.8×10^{-4}	3.8×10^{-5}

[a] All strains are *add* mutants, except for 8G5 (*add*$^+$).
[b] The transformation frequency is defined as the number of transformants per total number of viable cells.

transformants of mutants E6, E16, E34, M5, and M11 were obtained with either chromosomal DNA or plasmid DNA (Table 3). The complete lack of transformation is probably due to a deletion of a region spanning an *add* gene and a gene involved in competence, which is also closely linked to the *metD* marker (46). This is supported by the observation that the mutants E6, E16, E34, M5, and M11 were completely deficient in binding and uptake of transforming DNA (data not shown). The residual transformation of the other mutants with chromosomal DNA was between 1.5 and 3% of that of the wild-type strain (Table 3). However, with plasmid DNA, these mutants transformed to approximately 60% of that obtained in transformation of the wild type (Table 3), indicating that, in contrast to the transformation with chromosomal DNA, transformation with plasmid DNA was hardly affected by *add* mutations.

In all *add* mutants, the viability, defined as the number of CFU per A_{450} unit, was approximately 20% of that of the wild type (data not shown).

Sensitivity to MC and UV irradiation. The *add* mutants had lost their ability to form colonies on agar plates containing 30 ng of MC per ml, whereas the wild-type cells were still resistant to 75 ng of MC per ml. Mutant E6 was also more sensitive to UV irradiation than the wild-type strain but was much less sensitive than the *recE4* mutant (Fig. 1). The sensitivity of the other *add* mutants was similar to that of mutant E6 (data not shown).

Construction of a probe for *add* genes. To obtain a plasmid carrying *add* nucleotide sequences, the chromosomal DNA of mutant E6 was digested with *Pst*I. On pHV60, integrated in the chromosome of mutant E6, a unique *Pst*I site is located distal to an *Eco*RI site. After ligation at a low concentration of DNA (0.1 μg/ml) and transformation of an *E. coli* strain with the ligation mixture, tetracycline-resistant (Tc^r) transformants were selected (pHV60 carries a Tc^r marker) and plasmid DNA was isolated from the transformants. In this way, we were able to isolate a plasmid (pKV42) containing a 4.2-kb *Eco*RI-*Pst*I chromosomal DNA fragment. To examine whether this chromosomal DNA fragment contained part of the gene of interest, we transformed the mutants *add-5*, *add-71*, and *add-72* with plasmid pKV42 and tested the resulting Cm^r transformants for their sensitivity to MC. Approximately 90% of the Cm^r transformants obtained from transformation of *add-71* and *add-72* showed wild-type resistance to MC, whereas all of the Cm^r transformants of the *add-5* mutant remained sensitive to MC. As expected, the Cm^r marker in the Cm^r transformants was amplifiable, indicating that the vector had been integrated by a Campbell-like integration (data not shown). As far as tested, the MC-resistant transformants obtained in transformation of *add-71* and *add-72* also showed wild-type ADD activity (data not shown). The results indicate that the 4.2-kb *Eco*RI-*Pst*I chromosomal DNA fragment in pKV42 contained at least the promoter or the transcription terminator of an *add* transcription unit and that this DNA region overlapped both the *add-71* and *add-72* mutations but not the *add-5* mutation.

Screening of a *B. subtilis* chromosomal DNA bank in phage λEMBL4. Apparently, the 4.2-kb *Eco*RI-*Pst*I fragment obtained from mutant E6 carried at least the proximal or distal part of an *add* transcription unit. To isolate a larger DNA fragment, a gene bank of *B. subtilis* in phage λEMBL4, into which 12- to 17-kb *Eco*RI fragments of the *B. subtilis* chromosome had been inserted, was screened by using plasmid pKV42 as a probe. Recombinant phages whose DNA hybridized with the probe were isolated. Their DNAs all contained an *Eco*RI chromosomal DNA fragment of approximately 13.3 kb. This DNA fragment was isolated and inserted into the *Eco*RI site of the kanamycin resistance (Km^r) plasmid pGV1, which can replicate in both *E. coli* and

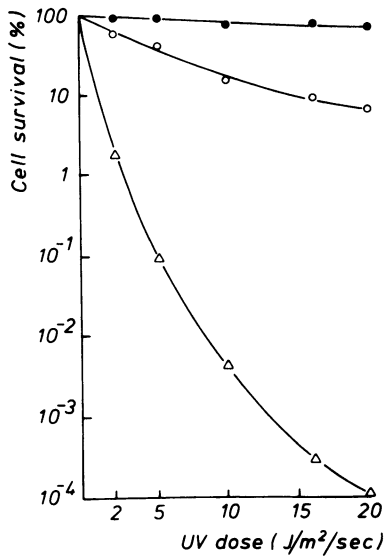

FIG. 1. UV survival of strains 8G5 (●), E6 (○), and 7G224 (△).

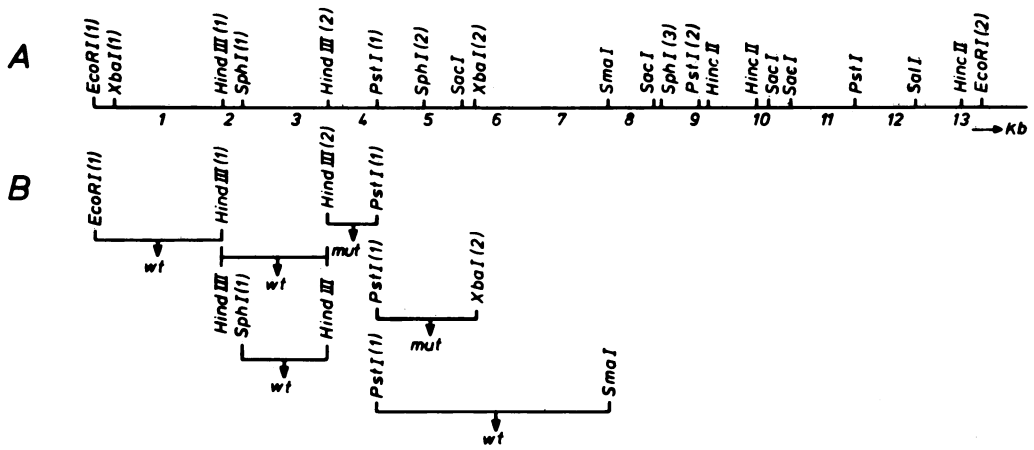

FIG. 2. Restriction map of the 13.3-kb *Eco*RI fragment and the localization of the *add* gene on this fragment. (A) Restriction map of the 13.3-kb *Eco*RI fragment. (B) The restriction fragments indicated were inserted into pUC plasmids. These plasmids were used to transform an 8G5 (*add*$^+$) strain of *B. subtilis*. The fragments which transformed the recipient to the mutant ADD phenotype (mut) and those which did not (wt) are indicated.

B. subtilis (47). This resulted in plasmid pGV133. The *add-5*, *add-71*, and *add-72* mutants were transformed with plasmid pGV133, and the resulting Km^r transformants were tested for their sensitivity to MC. Approximately 90% of the Km^r transformants of all three *add* mutants had the wild-type resistance to MC, indicating that the 13.3-kb *Eco*RI fragment contained a region, not only overlapping the *add-71* and *add-72* mutations but also the *add-5* mutation. Digestion of the 13.3-kb *Eco*RI fragment with *Eco*RI and *Pst*I resulted in the formation of five fragments with sizes of 4.9, 4.2, 1.8, 1.4, and 1.0 kb, respectively (data not shown). The 4.2-kb *Eco*RI-*Pst*I fragment was identical to that present in plasmid pKV42. The 4.9-kb fragment, a *Pst*I fragment, was inserted into pHV60, resulting in plasmid pKV49. Transformation of the *add-5* mutant with pKV49 resulted in transformants, approximately 85% of which showed wild-type resistance to MC. In contrast, in transformation of the mutants *add-71* and *add-72*, none of the transformants showed wild-type resistance to MC, indicating that the 4.9-kb *Pst*I fragment contained the region on which the *add-5* mutation was located.

Since the plasmids pKV42 and pKV49 contained only one chromosomal DNA fragment, transformation of the *add* mutants with these plasmids must have resulted from a Campbell-type integration into the chromosome. In addition, the wild-type ADD phenotype of the transformants indicates that both the 4.2-kb *Eco*RI-*Pst*I fragment and the 4.9-kb *Pst*I fragment should contain at least one end of a transcription unit involved in ADD synthesis.

Localization of the *add* transcription unit(s) on the 13.3-kb *Eco*RI fragment. To examine whether the 13.3-kb *Eco*RI fragment contained one complete, or perhaps more, *add* transcription units and to localize the transcription unit(s) on the 13.3-kb *Eco*RI fragment, we have chosen the following procedure.

Various restriction fragments of the 13.3-kb *Eco*RI fragment were inserted into the multiple cloning site of pUC plasmids. After insertion of the chromosomal DNA fragments, an additional *Eco*RI fragment, carrying the Em^r marker of plasmid pE194 (see Materials and Methods), which is expressed in *B. subtilis*, was inserted into the *Eco*RI site of the pUC plasmids. In transformation of a wild-type strain of *B. subtilis* with these plasmids, Em^r transformants will be formed as a result of Campbell-type integrations, and the Em^r transformants will have the ADD mutant phenotype if the plasmid contains an internal part of an *add* transcription unit. Wild-type ADD phenotypes will be obtained if the inserted DNA does not contain a part of an *add* transcription unit or if it contains at least one of the ends of the transcription unit. From Fig. 2, which shows the restriction map of the 13.3-kb *Eco*RI fragment and the capacity of the various restriction fragments in the pUC plasmids to produce transformants with the mutant or the wild-type ADD phenotype, it follows that only one complete transcription unit including the promoter and terminator is present on the 13.3-kb *Eco*RI fragment. This transcription unit is flanked by the *Sph*I(1) site on the left and the *Sma*I site on the right and is also present on the *Eco*RI(1)-*Sma*I fragment. This 7.7-kb *Eco*RI(1)-*Sma*I fragment was inserted into pGV1, resulting in plasmid pGV77.

Construction of an *add* deletion mutant and complementation. To obtain additional evidence that the entire transcription unit was present in an intact form on the *Eco*RI(1)-*Sma*I fragment and to examine whether it could complement a strain lacking this transcription unit, a *B. subtilis* strain was constructed in which the entire transcription unit was deleted. To that end, the *Sph*I(1)-*Sph*I(3) fragment was removed from the 13.3-kb *Eco*RI fragment in plasmid pGV133, resulting in a plasmid which contains an *Eco*RI(1)-*Sph*I-*Eco*RI(2) fragment of approximately 7.8 kb. The *Sph*I site was made blunt by T4 DNA polymerase and a *Hin*dII fragment, containing the Cm^r marker derived from pC194 (see Materials and Methods) which is expressed in *B. subtilis*, was inserted into the blunt-ended site, resulting in plasmid pGV93. This plasmid was linearized with *Bam*HI (present in the multiple cloning site of pGV1) to prevent Campbell-like integration. A wild-type strain of *B. subtilis* was transformed with this plasmid, and Cm^r transformants were selected. Since the plasmid contains two nonadjacent chromosomal DNA fragments in the same orientation as present on the chromosome, the plasmid will be integrated into the chromosome by a replacement recombination, resulting in the deletion of the *Sph*I(1)-*Sph*I(3) fragment, on which the entire *add* transcription unit is located. As expected, the resulting Cm^r transformants were sensitive to kanamycin and to MC and lacked ADD activity (data not shown). A number of these Cm^r transformants, denoted as 8G5 (*addA*) strains, were transformed with plasmid pGV77.

The resulting Km^r transformants were as resistant to MC as the wild-type strain and had wild-type ADD activity (data not shown). This indicates that the 7.7-kb *Eco*RI(1)-*Sma*I fragment is capable of complementing the deleted *add* transcription unit. This, together with the capacity of the various restriction fragments to produce ADD-positive and ADD-deficient phenotypes, as presented above, indicates that the entire *add* transcription unit is present in an intact form on pGV77.

Mapping of the *add-5*, *add-71*, and *add-72* mutations on the 13.3-kb *Eco*RI fragment. To determine where the various *add* mutations were located, a 5.4-kb *Xba*I(1)-*Xba*I(2) fragment (Fig. 2) was first inserted into pGV1, resulting in plasmid pGV54. The *add* mutants were transformed with this plasmid. The Km^r transformants from all strains had the wild-type ADD phenotype, indicating that the 5.4-kb *Xba*I fragment overlapped with all three *add* mutations. Since transformation with the *Pst*I(1)-*Pst*I(2) fragment inserted in pHV60 of *add-5* also produced wild-type transformants, but not in transformation of *add-71* and *add-72* (see previous section), it can be concluded that the *add-5* mutation is located on the *Pst*I(1)-*Xba*I(2) fragment and that the *add-71* and *add-72* mutations are located on the *Xba*I(1)-*Pst*I(1) fragment of the chromosome.

To map the *add-71* and *add-72* mutations more precisely, the 1.9-kb *Eco*RI(1)-*Hin*dIII(1) fragment and the 2.6-kb *Sph*I(1)-*Pst*I(1) fragment were inserted into pUC9 and pUC19, respectively. After the additional insertion of an *Eco*RI fragment, carrying the Em^r marker derived from pE194 (see Materials and Methods), into the *Eco*RI site of the pUC plasmids, the *add-71* and *add-72* mutants were transformed with these plasmids. Transformation of *add-71* and of *add-72* with pUC9 containing the *Eco*RI-*Hin*dIII fragment yielded Em^r transformants with the wild-type ADD phenotype, whereas transformation of these mutants with pUC19, containing the *Sph*I(1)-*Pst*I(1) fragment, yielded only ADD-deficient Em^r transformants. This indicates that the *add-71* and *add-72* mutations are located between the *Xba*I(1) site and the *Hin*dIII(1) site. Furthermore, these mutations are in an *add* transcription unit different from that in which the *add-5* mutation is located, since the transcription unit carrying the *add-5* mutation is located on the *Sph*I(1)-*Sma*I fragment (see above).

The following supports our conclusion that a second *add* transcription unit is present on the chromosome of *B. subtilis*; the probe obtained from mutant E6 contained the 4.2-kb *Eco*RI(1)-*Pst*I(1) fragment. This indicates that this mutation was produced by pHV60 integration to the left of the *Eco*RI(1) site in sequences specifying a second *add* transcription unit.

DISCUSSION

Insertional mutagenesis with pHV60 produced a considerable number of transformation-deficient, MC-sensitive mutants. These phenotypes suggest that the mutants were impaired in recombination. A surprisingly large fraction of these mutants (approximately 25%) were impaired in ADD activity, which may reflect that several genes are involved in ADD synthesis and that these genes may be of relatively large size (6).

In all *add* mutants, the viability was less than in the wild type, a characteristic which has also been observed in ADD-deficient mutants of other bacteria (4, 28).

Although the transformability of *add* mutants with chromosomal DNA was decreased to 1.5 to 3% of that of the wild-type, transformation with plasmid DNA was only slightly reduced. This strongly suggests that the efficiency of transformation with plasmid DNA is largely independent of ADD activity. In addition, the reduced frequency of transformation with chromosomal DNA cannot be attributed to reduced viability of the competent fraction of cells, because in that case transformation with plasmid DNA should be reduced to the same extent as that with chromosomal DNA. The role of the ADD enzyme in recombination is not yet clearly understood. Chaudhury and Smith (5) postulated that during conjugation in *E. coli*, the helicase activity of the enzyme is required for efficient recombination.

The cloned transcription unit involved in ADD synthesis in *B. subtilis* is located between the *Sph*I(1) site and the *Sma*I site (Fig. 2). This fragment should contain the complete transcription unit, because in transformation of a wild type with pUC plasmids carrying the *Sph*I(1)-*Hin*dIII(2) and the *Pst*I(1)-*Sma*I chromosomal DNA fragments, Em^r transformants with ADD wild-type phenotype were obtained.

The *add-5* mutation is located in the cloned transcription unit, whereas the *add-71* and *add-72* mutations are located in a different *add* transcription unit. Part of this second transcription unit is present on the *Eco*RI(1)-*Hin*dIII(1) fragment and should contain one of the ends of the transcription unit because in transformation of a wild-type strain with the plasmid pUC9, carrying the chromosomal *Eco*RI(1)-*Hin*dIII(1) fragment, the resulting Em^r transformants had the wild-type phenotype. The remaining part of the second transcription unit should be located to the left of the *Eco*RI site. The mutant E6, in which the entire 13.3-kb *Eco*RI region is present, lacks DNA to the left of the *Eco*RI(1) site as a result of replacement recombination of pHV60. This deleted region should contain the remaining part of a second *add* transcription unit and may contain additional genes involved in ADD synthesis. The ADD of other organisms was also been found to be encoded by more than one gene (43). Recently, it has been shown that it *E. coli* the ADD consists of three proteins (with molecular masses of 135, 125, and 58 kilodaltons), which are encoded by three genes (*recB*, *recC*, and *recD*) (1, 21). *recB* and *recD* are contiguous, but the *recC* gene is separated from the *recBD* genes by *ptr*, encoding a protease (1, 13–15).

Our results show that one of the ends of the cloned *B. subtilis* transcription unit is located between the *Sph*I(1) site and the *Hin*dIII(2) site, whereas one of the ends of the second transcription unit is located between the *Hin*dIII(1) site and the *Xba*I(1) site. Thus, it would seem that in *B. subtilis* the cloned transcription unit and the second *add* transcription unit are also separated from each other by an ADD-independent DNA sequence.

LITERATURE CITED

1. **Amundsen, S. K., A. F. Taylor, A. M. Chaudhury, and G. R. Smith.** 1986. *Rec*D: the gene for an essential third subunit of exonuclease V. Proc. Natl. Acad. Sci. USA **83**:5558–5562.
2. **Biswal, N., A. K. Kleinschmidt, H. C. Spatz, and T. A. Trautner.** 1967. Physical properties of the DNA of bacteriophage SP50. Mol. Gen. Genet. **100**:39–55.
3. **Bron, S., and G. Venema.** 1972. Ultraviolet inactivation and excision repair in *Bacillus subtilis*. I. Construction and characterization of a transformable eightfold-auxotrophic strain and two ultraviolet-sensitive derivatives. Mutat. Res. **15**:1–10.
4. **Capaldo-Kimball, F., and S. D. Barbour.** 1971. Involvement of recombination genes in growth and viability of *Escherichia coli* K-12. J. Bacteriol. **106**:204–212.
5. **Chaudhury, A. M., and G. R. Smith.** 1984. A new class of *Escherichia coli rec*BC mutants: implications for the role of recBC enzyme in homologous recombination. Proc. Natl. Acad.

Sci. USA **81**:7850–7854.
6. **Chestukhin, A. V., M. F. Shemyakin, N. A. Kalinina, and A. A. Prozorov.** 1972. Some properties of ATP dependent deoxyribonucleases from normal and *rec*-mutant strains of *Bacillus subtilis*. FEBS Lett. **24**:121–125.
7. **Clark, A. J.** 1973. Recombination deficient mutants of *Escherichia coli* and other bacteria. Annu. Rev. Genet. **7**:67–86.
8. **Dedonder, R. A., J.-A. Lepesant, J. Lepesant-Kejzlarová, A. Billault, M. Steinmetz, and F. Kunst.** 1977. Construction of a kit of reference strains for rapid genetic mapping in *Bacillus subtilis* 168. Appl. Environ. Microbiol. **33**:989–993.
9. **De Vos, W. M., and G. Venema.** 1981. Fate of plasmid DNA in transformation of *Bacillus subtilis* protoplasts. Mol. Gen. Genet. **182**:39–43.
10. **Doly, J., and C. Anagnostopoulos.** 1976. Isolation, subunit structure and properties of the ATP-dependent deoxyribonuclease of *Bacillus subtilis*. Eur. J. Biochem. **71**:309–316.
11. **Doly, J., D. LeRoscouet, and C. Anagnostopoulos.** 1981. Substrate specificity and adenosine triphosphatase activity of the ATP-dependent deoxyribonuclease of *Bacillus subtilis*. Eur. J. Biochem. **114**:493–499.
12. **Doly, J., E. Sasarman, and C. Anagnostopoulos.** 1974. ATP-dependent deoxyribonuclease in *Bacillus subtilis* and a mutant deficient in this activity. Mutat. Res. **22**:15–23.
13. **Dykstra, C. C., and S. R. Kushner.** 1985. Physical characterization of the cloned protease III gene from *Escherichia coli* K-12. J. Bacteriol. **163**:1055–1059.
14. **Dykstra, C. C., D. Prasher, and S. R. Kushner.** 1984. Physical and biochemical analysis of the cloned *recB* and *recC* genes of *Escherichia coli* K-12. J. Bacteriol. **157**:21–27.
15. **Finch, P. W., R. E. Wilson, K. Brown, I. D. Hickson, and P. T. Emmerson.** 1986. Complete nucleotide sequence of the *Escherichia coli ptr* gene encoding protease III. Nucleic Acids Res. **14**:7695–7703.
16. **Frischauf, A. M., H. Lehrach, A. Poustka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**:827–842.
17. **Goldmark, P. J., and S. Linn.** 1970. An endonuclease activity from *Escherichia coli* absent from certain *rec*$^-$ strains. Proc. Natl. Acad. Sci. USA **67**:434–441.
18. **Goldmark, P. J., and S. Linn.** 1972. Purification and properties of the *recBC* DNase of *Escherichia coli* K-12. J. Biol. Chem. **247**:1849–1860.
19. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
20. **Henner, D. J., and J. A. Hoch.** 1980. The *Bacillus subtilis* chromosome. Microbiol. Rev. **44**:57–82.
21. **Hickson, I. D., and P. T. Emmerson.** 1981. Identification of the *Escherichia coli rec*B and *rec*C gene products. Nature (London) **294**:578–580.
22. **Hoch, F. A., M. Barot, and C. Anagnostopoulos.** 1967. Transformation and transduction in recombination-defective mutants of *Bacillus subtilis*. J. Bacteriol. **122**:25–33.
23. **Hohn, B.** 1979. In vitro packaging of lambda and cosmid DNA. Methods Enzymol. **68**:299–309.
24. **Iordănescu, S.** 1975. Recombinant plasmid obtained from two different, compatible staphylococcal plasmids. J. Bacteriol. **124**: 597–601.
25. **Iordănescu, S.** 1977. Relationships between cotransducible plasmids in *Staphylococcus aureus*. J. Bacteriol. **129**:71–75.
26. **Ish-Horowicz, D., and F. J. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2999.
27. **Janniere, L., B. Niaudet, E. Pierre, and S. D. Ehrlich.** 1985. Stable gene amplification in the chromosome of *Bacillus subtilis*. Gene **40**:47–55.
28. **Kooistra, J., G. D. Small, J. K. Setlow, and R. Shapanka.** 1976. Genetics and complementation of *Haemophilus influenzae* mutants deficient in adenosine 5′-triphosphate-dependent nuclease. J. Bacteriol. **126**:31–37.
29. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
32. **Michel, B., B. Niaudet, and S. D. Ehrlich.** 1983. Intermolecular recombination during transformation of *Bacillus subtilis* competent cells by monomeric and dimeric plasmids. Plasmid **10**:1–10.
33. **Mulder, J. A., and G. Venema.** 1982. Isolation and partial characterization of *Bacillus subtilis* mutants impaired in DNA entry. J. Bacteriol. **150**:260–268.
34. **Nester, E., and J. Lederberg.** 1961. Linkage of genetics units of *Bacillus subtilis* in DNA transformation. Proc. Natl. Acad. Sci. USA **47**:52–55.
35. **Niaudet, B., A. Gose, and S. D. Ehrlich.** 1982. Insertional mutagenesis in *Bacillus subtilis*: mechanism and use in gene cloning. Gene **19**:277–284.
36. **Oishi, M.** 1969. An ATP-dependent deoxyribonuclease from *Escherichia coli* with a possible role in genetic recombination. Proc. Natl. Acad. Sci. USA **64**:1292–1299.
37. **Prozorov, A. A., N. A. Kalinina, L. S. Naumov, A. V. Chestukhin, and M. F. Shemyakin.** 1972. A mutant of *Bacillus subtilis* with impaired ability for recombination and reduced activity of the enzyme ATP-dependent deoxyribonuclease. Genetika **8**: 142–148.
38. **Rigby, P. W. J., M. Dieckman, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**: 237–251.
39. **Rosamond, J., K. M. Telander, and S. Linn.** 1979. Modulation of the action of the *recBC* enzyme of *Escherichia coli* K-12 by Ca^{2+}. J. Biol. Chem. **254**:8646–8652.
40. **Smith, H., W. DeVos, and S. Bron.** 1983. Transformation in *Bacillus subtilis*. Properties of DNA-binding-deficient mutants. J. Bacteriol. **153**:12–20.
41. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44**:1072–1078.
42. **Taylor, A., and G. R. Smith.** 1980. Unwinding and rewinding of DNA by the recBC enzyme. Cell **22**:447–457.
43. **Telander-Muskavitch, K. M., and S. Linn.** 1981. *Rec*BC-like enzymes: exonuclease V deoxyribonucleases, p. 233–250. *In* P. D. Boyer (ed.), The enzymes, vol. XIV. Academic Press, Inc., New York.
44. **Venema, G., R. H. Pritchard, and T. Venema-Schröder.** 1965. Fate of transforming deoxyribonucleic acid in *Bacillus subtilis*. J. Bacteriol. **89**:1250–1255.
45. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
46. **Vosman, B., J. Kooistra, J. Olijve, and G. Venema.** 1986. Integration of vector-containing *Bacillus subtilis* chromosomal DNA by a Campbell-like mechanism. Mol. Gen. Genet. **204**: 524–531.
47. **Vosman, B., J. Kooistra, J. Olijve, and G. Venema.** 1987. Cloning in *Escherichia coli* of the gene specifying the DNA-entry nuclease of *Bacillus subtilis*. Gene **52**:175–183.