JOURNAL OF BACTERIOLOGY, Apr., 1965
Vol. 89, No. 4

Localization of Spore Markers on the Chromosome of *Bacillus subtilis*

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Received for publication 7 December 1964

ABSTRACT

TAKAHASHI, I. (McMaster University, Hamilton, Ontario, Canada). Localization of spore markers on the chromosome of *Bacillus subtilis*. J. Bacteriol. **89**:1065–1067. 1965.—Attempts were made to locate spore markers on the chromosome by determining the mapping distance of *ery*^{r}, *str*^{r}, *phe*, *tyr*, and *ser* markers to which the spore markers are closely linked. The results obtained indicated that locus *sp N2-2* is located near the terminus and loci *sp 170-2*, *sp H12-4*, and *sp 1* are distributed between the middle and the origin of the chromosome. These spore markers are not linked to each other. The above observation confirms our previous finding that in *Bacillus subtilis* spore markers are distributed randomly along the chromosome.

Our transduction experiments have shown that genetic loci at which a mutation can affect sporulation in *Bacillus subtilis* are numerous, and that some spore markers are closely linked to various auxotrophic or antibiotic-resistance markers (Takahashi, 1965). These spore markers, therefore, appear to be distributed at random along the chromosome. Results obtained by Schaeffer (Acta Microbiol. Acad. Sci. Hung., *in press*) from transformation experiments also indicate that mutations concerning the formation of spores in the same organism are many and that they are not linked to one another. On the other hand, Spizizen, Reilly, and Dahl (1963) reported that spore markers are linked to a group of genes which control the synthesis of a proteolytic enzyme and a wall lytic enzyme to form a cluster.

It seems important, at this stage of progress, to establish a genetic map of the spore markers that have been described. Because all asporogenous mutants isolated in our laboratory are nontransformable under our experimental conditions and since no reliable methods for the quantitative determination of sporogenous recombinants are available, direct mapping of spore markers either by transformation or by transduction cannot be made. By making use of the fact that some of the spore markers are linked to auxotrophic or antibiotic-resistance markers, I have attempted to locate indirectly the spore markers on the chromosome. By use of the method of Yoshikawa and Sueoka (1963), a fairly accurate genetic map has been constructed from the mapping distance of the auxotrophic and antibiotic-resistance markers to which the spore markers are linked.

MATERIALS AND METHODS

Symbols. The following symbols are used: *ade*, adenine; *met*, methionine; *leu*, leucine; *ser*, serine; *phe*, phenylalanine; *tyr*, tyrosine; *his*, histidine; *ind*, indole; *thr*, threonine; *ery*^{r}, erythromycin resistance; *str*^{r}, streptomycin resistance; *sp*$^+$, sporogenous; *sp*$^-$, asporogenous.

Bacterial strains. A wild-type strain, W23SE, derived from strain W23 (Yoshikawa and Sueoka, 1963) was the source of deoxyribonucleic acid (DNA). Strain W23SE was resistant to streptomycin (1,000 μg/ml) and to erythromycin (1 μg/ml). Strain AM (*ade*$^-$, *met*$^-$), derived from strain Mu8u5u6 (*ade*$^-$, *leu*$^-$, *met*$^-$) of Yoshikawa and Sueoka (1963) by transformation, was used as reference in the determination of relative marker frequencies. Strains N2 (*sp*$^+$, *ser*$^-$) and H12 (*sp*$^+$, *phe*$^-$) were derived, by ultraviolet irradiation, from a wild-type strain (W) obtained from C. Anagnostopoulos. Strain 170 (*sp*$^+$, *tyr*$^-$) was isolated by plating cells of strain W with Tryptose Blood Agar Base (TB agar Difco), containing 100 μg/ml of streptomycin.

Preparation of DNA. Crude DNA preparations from exponentially growing cells (DNA I) were obtained as follows. Cells of W23SE were grown in Penassay Broth (Difco) for 3 hr on a water-bath shaker. The culture was diluted 10 times in the above medium, and the incubation was continued for an additional 1.5 hr. To stop further DNA replication, cultures were heated at 60 C for 10 min and chilled in ice water. Cells were collected by centrifugation at 5,000 × *g* for 15 min and were resuspended in SSC (0.15 M NaCl + 0.015 M sodium

citrate, pH 7.0). The cell suspension was incubated with lysozyme (100 μg/ml) for 30 min to lyse the cells. Crude DNA fibers were collected from the lysozyme lysate by the addition of alcohol, and were dissolved in SSC containing 0.45% sodium lauryl sulfate by shaking at room temperature. The shaking continued for an additional 2 hr, and solid NaCl was added to a concentration of 1 M. The solution was then centrifuged at 27,000 × *g* for 30 min to sediment denatured protein. The addition of alcohol to the supernatant fluid resulted in the formation of fibers which were practically free from protein, but still contained ribonucleic acid. The fibers were stored in 70% alcohol for 2 days in the cold and dissolved in SSC. Samples of DNA from cells in the stationary phase (DNA II) were prepared from 18-hr cultures in Penassay Broth by the same extraction procedure.

Transformation technique. Transformation experiments were carried out as follows. Recipient cells grown on TB agar were inoculated into Penassay Broth and incubated with shaking for 4 hr. The culture was diluted 10 times with a minimal medium (Spizizen, 1958) supplemented with 0.01% each of yeast extract (Difco) and Casamino Acids (Difco), and was incubated with shaking for 90 min. Samples of DNA (0.02 μg/ml, final concentration) were added to the culture and incubated with shaking for 30 min. The reaction was terminated by the addition of deoxyribonuclease (10 μg/ml). Prototrophic transformants were scored on the minimal agar of Spizizen (1958). When double auxotrophs were used as recipients, transformants were scored on the above agar medium containing appropriate amino acids at the concentration of 25 μg/ml. Transformation of antibiotic-resistance markers was carried out as described previously (Takahashi, 1965). To detect streptomycin-resistant transformants, TB agar containing 2,000 μg/ml of streptomycin sulfate was used as overlaying agar. The numbers of prototrophic or antibiotic-resistant transformants were counted after 2 days. All incubations in this study were at 37 C, and centrifugations were at 5 C, unless otherwise stated.

Mapping technique. Assuming that the chromosome replicates semiconservatively from one end (origin) and proceeds continuously toward the other end (terminus) at an approximately uniform speed, Yoshikawa and Sueoka (1963) were able to obtain a linear genetic map of *B. subtilis* by the transformation technique. According to their model, in an exponentially growing population without synchrony, the frequency of chromosomes whose replication has just started is nearly twice as high as that of chromosomes which are about to complete their replication. This would imply that the frequency of a genetic marker in an exponentially growing population is a unique function of its location on the chromosome. The fre-

TABLE 1. *Relative frequencies of various markers in DNA of Bacillus subtilis in exponential- and stationary-growth phases*

Recipient culture	DNA*	Transformants per 0.1 ml		Transformants (met^+)	Mapping function
AM	I	met^+	473		
		ade^+	1,010	2.14	2.02
AM	II	met^+	1,035		
		ade^+	1,092	1.06	
N2	I	ser^+	98	0.21	1.17
	II	ser^+	188	0.18	
170	I	tyr^+	4,080	8.63	1.41
	II	tyr^+	6,350	6.14	
H12	I	phe^+	4,890	10.34	1.59
	II	phe^+	6,720	6.49	

* DNA I was prepared from cells of W23SE in the exponential-growth phase, and DNA II was prepared from those of the stationary phase.

TABLE 2. *Relative frequencies of str^r and ery^r markers in DNA of Bacillus subtilis in exponential- and stationary-growth phases**

DNA†	Transformants per 0.1 ml		Transformants (met^+)	Mapping function
I	met^+	435		
	ade^+	766	1.76	1.91
II	met^+	1,232		
	ade^+	1,138	0.92	
I	eyr^r	628	1.44	1.80
II	eyr^r	986	0.80	
I	str^r	125	0.29	1.71
II	str^r	214	0.17	

* Strain AM was used as recipient.

† DNA I was prepared from cells of W23SE in the exponential-growth phase, and DNA II was prepared from those of the stationary phase.

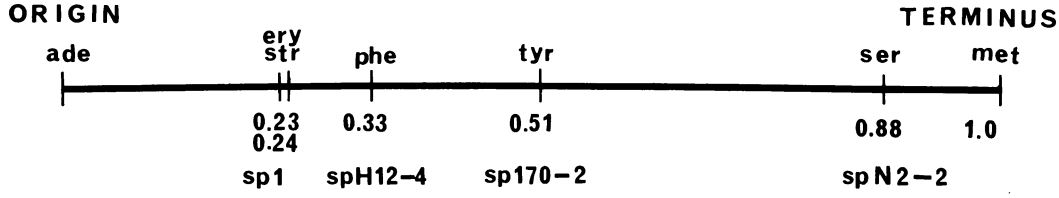

FIG. 1. *Genetic map showing the location of amino acid markers, antibiotic markers, and spore markers on the chromosome of Bacillus subtilis.*

quency distribution of markers as a function of their position, X, on the chromosome is $g(X) = 2^{1-x}$. The function $g(X)$ is designated as the mapping function. In our experiment, the numbers of transformants for each marker were normalized to the number of met^+ transformants. The normalized values obtained with DNA I were then normalized again to the values obtained with DNA II. The final values (mapping function) were used to calculate the mapping distance, X.

RESULTS AND DISCUSSION

It was reported previously that auxotrophic markers and spore markers are jointly transduced at high frequencies in strains Sp⁻N2-2 (*sp*⁻, *ser*⁻), Sp⁻170-2 (*sp*⁻, *tyr*⁻), and Sp⁻H12-4 (*sp*⁻, *phe*⁻). It was also found that a spore marker (*sp 1*) was linked to *ery*^{r} and *str*^{r} loci in strain Sp⁻1 (Takahashi, 1965). To locate indirectly these spore markers on the chromosome, mapping of the above amino acid markers and antibiotic-resistance markers was carried out by the above-mentioned technique. Since the asporogenous mutants were nontransformable, strains N2 (*sp*$^+$, *ser*⁻), 170 (*sp*$^+$, *tyr*⁻), and H12 (*sp*$^+$, *phe*⁻), from which the asporogenous mutants were derived, were employed as recipients. Strains N2, 170, and H12 were sporogenous and transformable for respective auxotrophic markers. Strain AM was used for the determination of mapping distances of *ery*^{r} and *str*^{r} markers.

The mapping functions for amino acid markers studied are listed in Table 1 and those for antibiotic markers, in Table 2. Data presented in these tables were obtained from a single experiment. The mapping distance (X) for each marker was calculated with average mapping functions obtained from four independent experiments.

As expected, *ade*, which is located at the origin, was twice as frequent as *met*, which is located at the terminus, when W23SE was used as donor (Table 1). This result is in agreement with that of Yoshikawa and Sueoka (1963).

Intermediate mapping functions were obtained with markers other than *ade* and *met*. From these values, the mapping distance for each marker was calculated and a genetic map was constructed (Fig. 1). As shown in Fig. 1, *ser* was found to be near the terminus and other markers were distributed between the middle and the origin of the chromosome.

The mapping distances of *str*^{r} and *ery*^{r} were very similar, suggesting that these two markers might be closely linked. In fact, when erythromycin-resistant colonies obtained from strain AM by transformation with W23SE as donor (Table 2) were examined, 48% (68 of 143) of them were resistant to streptomycin. Of the streptomycin-resistant transformants, 45% (71 of 159) were also resistant to erythromycin. These two markers are therefore linked with each other on the chromosome of *B. subtilis*. A similar situation was reported for the *tyr-ind-his* linkage group by Yoshikawa and Sueoka (1963). Thus, the spore markers studied here should have a mapping distance similar to that of amino acid markers or antibiotic markers to which they are linked. Approximate locations of the spore markers on the chromosome are presented in Fig. 1.

In *Escherichia coli*, *Salmonella typhimurium*, and *B. subtilis*, genes controlling related functions, are, as a rule, closely associated to form a cluster (Demerec, 1964). The foregoing data, however, clearly indicate that genes controlling the formation of spores are not clustered in a small region of the chromosome. Spore markers studied here are located on the chromosome far apart from each other, although a mutation at any of these loci would result in the expression of the asporogenous phenotype (inability to form mature spores). The interpretation of this result may be that there exist a number of independent biochemical processes which participate in the formation of spores, and that a block in any one of these processes may prevent the formation of mature spores. The data presented in this paper, therefore, confirm the previous finding made in transduction experiments (Takahashi, 1965).

ACKNOWLEDGMENT

This work was supported by a grant in aid of research (A-1996) from the National Research Council of Canada.

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