Mapping the δ -Aminolaevulinic Acid Synthetase Locus in *Bacillus subtilis*

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SUMMARY

HemA mutants of *Bacillus subtilis* 168 requiring δ -aminolaevulinic acid for their growth were isolated either following selection with streptomycin or by exposure to copper sulphate, and studied in reciprocal transduction experiments. Both groups of mutants were found to be genetically identical and the *hemA* locus was unlinked to a streptomycin resistance marker; it was linked to the group III markers mapped by Dubnau, Goldthwaite, Smith & Marmur (1967) and is located between the *ilvA1* and *phe-1* loci.

INTRODUCTION

Bacillus subtilis strain 168 exposed to either copper sulphate or streptomycin gives mutants defective in protohaem synthesis (Anderson & Ivánovics, 1967). For routine isolation of such mutants streptomycin selection was convenient and effective (Marjai, Kiss & Ivánovics, 1970). One class of mutants (*hemA*) required δ -aminolaevulinic acid (ALA) for growth and were apparently deficient in δ -aminolaevulinic acid synthetase activity. The aim of this work was to determine the location of the *hemA* locus in the genome of *Bacillus subtilis* 168 in relation to the genetical markers described by Dubnau *et al.* (1967).

METHODS

Bacterial strains. The results of preliminary experiments suggested that *hemA* was located somewhere in the third linkage group of Dubnau *et al.* (1967). Only strains with reference markers in this linkage group and their *hemA* derivatives obtained by transformation were therefore used. Table 1 lists the strains used for mapping the *hemA* locus, except for a number of mutants with ALA requirement which were isolated from SZ1 and from a streptomycin-resistant derivative of it and used in experiments for the characterization of the *hemA* locus. These strains, *try-2hemA2str*, *try-2hemA16str*, and *try-2hemA17str* were isolated from a mutant resistant to $> 500 \mu\text{g./ml.}$ streptomycin following exposure to copper sulphate. Strains *try-2hemA6* and *try-2hemA8* were obtained by streptomycin selection (Marjai *et al.* 1970).

Phages. Phage 3NT (Ivánovics & Csiszár, 1962) and PBS-1 (Takahashi, 1961) were used for transduction. Lysates were prepared and assayed as described previously (Csiszár & Ivánovics, 1965).

Media. Yeast extract peptone medium (YP) was the complete nutrient medium. Glutamate glycerol medium (GGM) was prepared as described by Anderson & Ivánovics (1967), and supplemented with specific growth requirements when appropriate: $2 \mu\text{g./ml.}$ ALA, $50 \mu\text{g./ml.}$ tryptophan and $30 \mu\text{g./ml.}$ other amino acids. Solid media contained 1.5% (w/v) agar.

Transduction. Recipient bacteria grown overnight in YP medium were diluted 1:50 in the same medium, aerated by shaking in a water bath at 37°, and grown to an extinction of 0.45 at 620 nm. equivalent to 2×10^8 bacteria/ml. For *hemA* mutants YP medium was supplemented with ALA. Transducing phage was added at a multiplicity of infection of one, the mixture kept at room temperature for 30 min. and then centrifuged for 15 min. at 2500 rev./min. in an angle centrifuge, the pellet was then resuspended in the same volume of GGM. Recombinants were selected on appropriately supplemented GGM plates and scored for unselected markers by replica plating to other media either directly or following restreaking on selective medium (both methods gave identical results).

Table 1. *Strains used to map the hemA locus in Bacillus subtilis 168*

Strain	Previous name	Genotype	Source
SZ 1	R12	<i>try-2</i>	P. Schaeffer
SZ 2	—	Prototroph	By transformation of SZ 1
SZ 3	R15	<i>try-2 hemA1</i>	T. J. Anderson & G. Ivánovics (1967)
SZ 4	GSY 384	<i>argA2 leu-1</i>	C. Anagnostopoulos
SZ 5*	—	<i>argA2 leu-1 hemA1</i>	SZ 4 <i>argA2 leu-1</i> → SZ 3
SZ 6*	—	<i>argA2 hemA1</i>	SZ 4 <i>argA2</i> → SZ 3
SZ 7*	—	<i>leu-1 hemA1</i>	SZ 4 <i>leu-1</i> → SZ 3
SZ 8	GSY 244	<i>phe-1 ilvA1</i>	C. Anagnostopoulos
SZ 9*	—	<i>phe-1 hemA1</i>	SZ 3 <i>hemA1</i> → SZ 8
SZ 10*	—	<i>ilvA1 hemA1</i>	SZ 3 <i>hemA1</i> → SZ 8
SZ 11	—	<i>try-2 str</i>	Str-resistant from SZ 1
SZ 12	—	<i>try-2 hemA1 str</i>	Str-resistant from SZ 3
SZ 13	—	<i>try-2 nia-60</i>	From SZ 3 by nitrosoguanidine treatment
SZ 14*	—	<i>hemA1</i>	SZ 2 → SZ 3

* Strains constructed by transformation of the strains indicated. The arrows indicate the relevant donors.

RESULTS

Analysis of the hemA locus

The copper sulphate method only yielded mutants with a protohaem deficiency when a streptomycin-resistant mutant of strain 168 was used. So far, all attempts to obtain such mutants from streptomycin-sensitive bacteria have failed. When haem mutants were isolated from streptomycin-sensitive 168 bacteria following streptomycin selection, a fairly high concentration (100 to 200 µg./ml.) was used. Nevertheless, the haem dependency was associated with only a small increase in the streptomycin resistance of the mutants which was lost upon spontaneous reversion to haem independency (Marjai *et al.* 1960). The mutants could not be classed as conditionally streptomycin-dependent (Gorini & Kataja, 1964), since streptomycin did not substitute for the requirement of the auxotroph. It therefore seemed likely that the haem requirement was not associated with an alteration of the streptomycin locus, although the observations referred to above might indicate some relation between streptomycin and haem markers.

To test whether the *str* and *hemA1* markers were linked or not, strain SZ 3 (*try-2 hemA1*) was transduced with phage 3 NT propagated on strain SZ 11 (*try-2 str*) and the selected *hemA1*⁺ transductants screened for streptomycin resistance by replication. None of 800 colonies was streptomycin-resistant. Also, when strain SZ 1 was transduced with SZ 12 lysates no co-transduction of *str* and *hemA1* markers was obtained. In this case, the transductants were selected for streptomycin resistance on plates containing tryptophan, ALA, + streptomycin, and replicated to GGM plates containing only tryptophan.

In reciprocal transduction crosses made with *hemA* mutants isolated either by copper sulphate or streptomycin treatment very few or no transductants were obtained. These results indicate that a single gene codes for δ -aminolaevulinic acid synthetase.

Mapping of the *hemA* locus

Two-factor crosses. Since one of our aims was to use phage 3 NT instead of PBS-1 and our transduction technique was not the same as that of Dubnau *et al.* (1967), transduction with the two phages was compared using the technique developed in this laboratory (Csiszár & Ivánovics, 1965). All recipients used possessed the standard markers of Dubnau *et al.* (1967). Both phages, PBS-1 and 3 NT, gave identical results (see Table 2). Hoch, Barat & Anagnostopoulos (1967) also obtained the same results in comparative transduction tests with these two phages and recombination-defective mutants of *Bacillus subtilis*. Recombination frequencies were identical for all markers tested, and corresponded well with the values obtained by Dubnau *et al.* (1967) except for *leu*⁺ transductants of sz4. On minimal medium supplemented only with arginine, the frequency of recombinants did not exceed 10 %, but on the sequential addition of amino acids except leucine to GGM + arginine there was a gradual increase in recombination which reached a peak value of 65.4 % when all (19) amino acids had been added.

Table 2. *Recombination between standard reference markers in transduction with phages 3 NT and PBS-1*

Recipient	Phage*	Selected marker	Total no. of transductants	Recombinants		
				Class	No. of colonies	%
sz8 (<i>phe-1 ilvA1</i>)	3NT	<i>ilv</i> ⁺	1547	<i>phe ilv</i> ⁺	342	22.1
		<i>phe</i> ⁺	1579	<i>phe</i> ⁺ <i>ilv</i>	381	24.1 (22)
	PBS-1	<i>ilv</i> ⁺	1217	<i>phe ilv</i> ⁺	288	23.6
		<i>phe</i> ⁺	1660	<i>phe</i> ⁺ <i>ilv</i>	390	23.5
sz4 (<i>argA2 leu-1</i>)	3NT	<i>arg</i> ⁺	1138	<i>leu arg</i> ⁺	809	71.1 (73)
		<i>leu</i> ⁺ †	570	<i>leu</i> ⁺ <i>arg</i>	373	65.4 (75)

$$\text{Recombination \%} = \frac{\text{no. of recombinant colonies}}{\text{total no. of transductants}} \times 100.$$

Numbers in brackets indicate the data of Dubnau *et al.* (1967).

* Phage lysates were prepared on sz2 (prototroph) donor bacteria.

† All amino acids except leucine were included in the GGM medium.

To establish the linkage between different standard markers and *hemA1* in the recipients, a series of crosses were made with phage 3 NT propagated either on prototrophic or on sz3 donor bacteria. The results of these two-point crosses appear in Table 3. Agreement between the percentage recombination values obtained with either of the two selected markers was satisfactory in all cases except when strain sz6 was the recipient. The same number of bacteria from the same transduction mixture was plated on each selective medium, but there was a difference between the total number of transductants obtained in each case. In contrast, the number of *arg*⁺*hem*⁺ transductants was almost identical on both media. Either the *hemA1*⁺ marker alone of the donor did not recombine with the recipient chromosome, or if it did, some of the recombinants were unable to form colonies. The phenomenon was not studied further, and an 88.4 % recombination value was taken for the sz6 × sz2 cross in constructing a linkage map.

Table 3. Two-factor crosses with *hemA1* and reference markers in transduction with phage 3NT

Recipient	Donor	Selected marker*	Total no. of transductants	Recombinants		
				Class	No. of colonies	%
SZ9 (<i>phe-1 hemA1</i>)	SZ2 (prototroph)	<i>phe</i> ⁺	375	<i>phe</i> ⁺ <i>hem</i>	79	21.0
		<i>hem</i> ⁺	331	<i>phe hem</i> ⁺	58	17.5
SZ10 (<i>ilvA1 hemA1</i>)	SZ2	<i>ilv</i> ⁺	785	<i>ilv</i> ⁺ <i>hem</i>	64	8.1
		<i>hem</i> ⁺	927	<i>ilv hem</i> ⁺	90	9.7
SZ6 (<i>argA2 hemA1</i>)	SZ2	<i>arg</i> ⁺	699	<i>arg</i> ⁺ <i>hem</i>	618	88.4
		<i>hem</i> ⁺	232	<i>arg hem</i> ⁺	145	62.5†
SZ7 (<i>leu-1 hemA1</i>)	SZ2	<i>leu</i> ⁺	1091	<i>leu</i> ⁺ <i>hem</i>	336	30.8
		<i>hem</i> ⁺	415	<i>leu hem</i> ⁺	126	30.4
SZ3 (<i>try-2 hemA1</i>)	SZ13 (<i>try-2 nia-60</i>)	<i>hem</i> ⁺	563	<i>hem</i> ⁺ <i>nia</i> ⁺	187	33.2

* When *hem*⁺ transductants were selected, GGM agar was supplemented with 0.05% vitamin-free casein hydrolysate (Difco).
† Identical numbers of bacteria were plated; the low number of colonies probably indicates that some transductants did not form colonies.

The results of two-factor crosses gave some information on the distance between individual markers and also gave an indication of their sequence, but the markers studied could not be ordered solely on the basis of this data. Consequently a series of three-factor crosses were performed.

Three-factor crosses. In all, six markers were examined in three separate experiments, the results of which are presented in Table 4. In three-factor crosses the class with the lowest number of colonies was taken to represent recombinants in which four cross-overs had occurred. A diagrammatic representation of the possible order of markers in the case of four cross-overs is shown in Fig. 1.

Fig. 1. The classes of recombinants involving four cross-overs according to the sequence of markers in the three-point tests of Table 4.

Fig. 2. The position of *hemA1* locus in linkage group III of *Bacillus subtilis*.

The results of Expt. 1 (Table 4) show that *hemA1* is located between *ilvA1* and *phe-1*, while Expt. 3 indicates that *phe-1* is located between *ilvA1* and *nia-60*. This is in agreement with the results of the two-point crosses (Table 3) and suggests the order *ilvA1-hemA1-phe-1-nia-60*. On the basis of Expt. 2 and the results of the two-factor crosses, i.e. SZ 4 $\times$ SZ 2, SZ 7 $\times$ SZ 2, and SZ 6 $\times$ SZ 2, carried out with phage 3 NT (Tables 3, 4) *leu-1* seems to be located between *argA2* and *hemA1*. The first marker in linkage group III of *Bacillus subtilis* 168 is *arg* according to Dubnau *et al.* (1967) and *argA2* is weakly linked to *hemA1* (see SZ 6 $\times$ SZ 2, Table 3). The results of crosses SZ 7 $\times$ SZ 2 and SZ 10 $\times$ SZ 2 indicate that *ilvA1* is nearer to *hemA1* than *leu-1* is to *hemA1*. The results of two and three-factor crosses combined suggest

Table 4. *Three-factor crosses with hemA1 and reference markers using transduction with phage 3 NT*

Experiment	Recipient	Donor	Selected marker	Total no. of colonies tested	Genotype of classes	No. of colonies
1	SZ 8 (<i>phe-1 ilvA1</i>)	SZ 3 (<i>try-2 hemA1</i>)	<i>phe</i> ⁺	520	<i>phe</i> ⁺ <i>hem</i> ⁺ <i>ilv</i> ⁺	19
					<i>phe</i> ⁺ <i>hem</i> ⁺ <i>ilv</i> ⁺	249
					<i>phe</i> ⁺ <i>hem</i> ⁺ <i>ilv</i>	119
					<i>phe</i> ⁺ <i>hem</i> <i>ilv</i>	133
			<i>ilv</i> ⁺	342	<i>ilv</i> ⁺ <i>hem</i> ⁺ <i>phe</i> ⁺	13
					<i>ilv</i> ⁺ <i>hem</i> <i>phe</i> ⁺	227
					<i>ilv</i> ⁺ <i>hem</i> ⁺ <i>phe</i>	56
2	SZ 5 (<i>argA2 leu-1 hemA1</i>)	SZ 2 (prototroph)	<i>arg</i> ⁺	1338	<i>ilv</i> ⁺ <i>hem</i> <i>phe</i>	46
					<i>arg</i> ⁺ <i>leu</i> ⁺ <i>hem</i> ⁺	90
					<i>arg</i> ⁺ <i>leu</i> <i>hem</i> ⁺	18
					<i>arg</i> ⁺ <i>leu</i> ⁺ <i>hem</i>	773
			<i>hem</i> ⁺	1054	<i>arg</i> ⁺ <i>leu</i> <i>hem</i>	457
					<i>hem</i> ⁺ <i>leu</i> ⁺ <i>arg</i> ⁺	314
					<i>hem</i> ⁺ <i>leu</i> <i>arg</i> ⁺	23
3	SZ 8 (<i>phe-1 ilvA1</i>)	SZ 13 (<i>try-2 nia-60</i>)	<i>hem</i> ⁺	646	<i>hem</i> ⁺ <i>leu</i> ⁺ <i>arg</i>	646
					<i>hem</i> ⁺ <i>leu</i> <i>arg</i>	71
					<i>ilv</i> ⁺ <i>phe</i> ⁺ <i>nia</i> ⁺	128
			<i>ilv</i> ⁺	314	<i>ilv</i> ⁺ <i>phe</i> <i>nia</i> ⁺	112
					<i>ilv</i> ⁺ <i>phe</i> ⁺ <i>nia</i>	66
					<i>ilv</i> ⁺ <i>phe</i> <i>nia</i>	8

that the order of the loci is *argA2-leu-1-ilvA1-hemA1-phe-1-nia-60*. This order and the recombination values for the reference markers are in good agreement with those described by Dubnau *et al.* (1967) in constructing a genetic map of *B. subtilis* 168. Thus the position of the *hemA1* gene controlling δ -aminolaevulinic acid synthetase was located in linkage group III between *ilvA1* and *phe-1* (Fig. 2). The *nia-60* locus of strain SZ 13 is located to the right of the other markers studied. Whether the *nia-60* marker or our mutant was identical to the *nia* markers of Dubnau *et al.* (1967) is uncertain, but its position is similar.

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