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Genetic Mapping of a Mutant Defective in D, L-Alanine Racemase in *Bacillus subtilis* 168

MICHAEL J. DUL AND FRANK E. YOUNG

Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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Genetic analysis of a D-alanine requiring mutant (*dal*) of *Bacillus subtilis* reveals that the gene that codes for D,L-alanine racemase is linked to *pur*B. The order of genes in this region of the chromosome is *pur*B, *pig*, *tsi*, *dal*. Thus there are at least two clusters of genes that regulate cell wall biosynthesis in *B. subtilis*.

Analysis of the genetic map of *Bacillus subtilis* reveals that the genes involved in the biosynthesis of teichoic acid, glucosylation of teichoic acid, and flagella are linked to the *his*A locus (9). Genetic maps of other microorganisms, such as *Escherichia coli* (6, 8) and *Salmonella* (7), have more than a single cluster of genes regulating cell wall synthesis. This note reports the localization of a genetic locus involved in the synthesis of D,L-alanine racemase (EC 5.1.1.1). The enzyme is required for one of the earliest steps in the production of the D-Ala-D-Ala dipeptide. Thus it is essential for the synthesis of the uridine 5'-diphosphate-*N*-acetyl-muramyl-pentapeptide and the continued production of peptidoglycan.

The chromosomal location of the D,L-alanine racemase (*dal*) locus was accomplished by transduction with phage PBS1. Donor cells for transduction were shaken for 3 h in Antibiotic Medium 3 (Penassay Broth, Difco) at 37 C and then mixed with PSB1 so that the final multiplicity of infection was 10:1. The mixture was incubated at 250 rpm for 1 h at 37 C, chloramphenicol was added to a final concentration of 5 μg/ml, and shaking was continued for 2 h. After incubation for 12 to 18 h at 37 C, the donor lysate was treated with deoxyribonuclease (50 μg/ml) and filtered through a type HA filter (0.45 μm pore size, Millipore Corp., Bedford, Mass.). Recipients for transduction were streaked into 3.0 ml of 0.3% soft agar on a tryptose blood agar base plate (TBAB, Hospital Service Technology, Boston, Mass.) to allow motile cells to swarm. Because PBS1 adsorbs only to motile cells (4), highly motile variants were selected as recipients. A loopful of cells was inoculated into 10 ml of Penassay Broth and shaken at 37 C until maximal motility was achieved, as determined by phase microscopy. A sample (0.5 ml) of the motile, recipient cells was mixed with an equal volume of the donor lysate, and incubated at 250 rpm for 15 min at 37 C. The mixture was diluted to 5.0 ml with Spizizen's minimal salts (1), centrifuged at 8,000 × *g* for 10 min, and the cells were resuspended in 1.0 ml of Spizizen's minimal salts solution. Samples (0.1 ml) were plated onto selective medium: TBAB (Difco) for *dal*$^+$ recombinants and Spizizen's minimal agar (1) supplemented with 0.5% neutralized casein hydrolysate for *pur*$^+$ recombinants. The nomenclature of the established markers used in all tables is identical to that of Young and Wilson (9).

The original *dal* mutant (60935) was obtained from Ernst Freese and is one of several *dal* strains described by Freese et al. (2). The mutant locus was transferred into an isogenic background prior to genetic mapping. The standard recipient background is strain BR151 carrying *lys-3*, *trpC2*, and *metB10*. A compilation of the strains utilized to map the *dal* locus and their origin is shown in Table 1. Two-factor crosses demonstrate that the *dal* locus is cotransduced with the three markers in the *pur*B linkage group (Table 2). The position of the *dal* locus was established by three-factor crosses. The first cross was designed to confirm the order of the reference markers in the *pur*B region of the chromosome in isogenic strains (Table 3). The data show that the order is *pur*B, *pig*, *tsi*. The second cross was performed to localize unequivocally the position of the *dal* locus. The order of the genes is *pur*B, *pig*, *dal* (Table 4). It can also be inferred from these data that the new locus is quite distant to the two reference markers used in this cross. The *dal*

TABLE 1. *Summary of strains*

Strain	Genotype designation	Source
60935	*dal metB*	E. Freese
MB 227	*pig*	J. Marmur
MB 502	*tsi*	E. Sigel
RUB 783	*purB hisA metB10 leu-8*	U. Streips
RUB 1200	*purB metB10 trpC2*	D. J. Groves
RUB 1401	*purB metB10 trpC2 lin*^{R}	
RUB 1402	*dal hisA metB10 leu-8*[a]	M. Dul
RUB 1404	*purB pig hisA leu-8*[a]	M. Dul

[a] Constructed in this laboratory by congression with RUB 783.

TABLE 2. *Linkage of dal to other markers by transduction*[a]

Unselected donor marker	Selected marker	Recombinants[b]	Cotransfer (%)
purB (RUB 1200)	*dal*$^+$	29/320	9.1
purB (RUB 1401)	*dal*$^+$	16/190	8.4
pig (MB 227)	*dal*$^+$	71/311	22.8
tsi (MB 502)	*dal*$^+$	44/208	22.1

[a] The recipient in all the crosses was RUB 1402.
[b] Proportion of the number of recombinants carrying the unselected marker.

TABLE 3. *Linkage of markers to purB in three-factor crosses*

Type of recombinants[a]			No. of recombinants
purB	*pig*	*tsi*	
1	1	1	72
1	1	0	300
1	0	0	98
1	0	1	0

[a] Selection was for *purB*$^+$ recombinants. The donor was MB 502 and the recipient was RUB 1404. The designation 1 and 0 refer to donor and recipient genotypes, respectively.

TABLE 4. *Three-factor analysis of linkage in dal pig and purB*

Type of recombinants[a]			No. of recombinants
dal	*pig*	*purB*	
1	0	0	253
1	0	1	0
1	1	0	29
1	1	1	12

[a] Selection was for *dal*$^+$. The donor was RUB 1404 and the recipient was RUB 1402. The designation 1 and 0 refer to donor and recipient genotypes, respectively.

locus could not be linked either to lincomycin resistance or to the gene involved in the metabolism of trehalose. These markers are proximal and distal to the segment of the genome carrying the *pur*B region. Thus, the *dal* locus still does not permit the genetic linkage of the *pur*B segment with the rest of the genetic map of *B. subtilis*. In view of the study of Gyurasits and Wake (3), which demonstrates that the circular chromosome of *B. subtilis* has a region of bidirectional replication, it is possible that the portion of the genome of *B. subtilis* containing the genes linked to *pur*B may reside in a different location from that currently shown (F. E. Young and G. A. Wilson, *In* R. C. King (ed.), Handbook of Genetics, in press).

The localization of D,L-alanine racemase is important for several reasons. First, it establishes a second locus of genes involved in cell wall biosynthesis in an area of the genome that to date has not been mapped extensively. This presents the opportunity to find other mutants in this region by transformation using nitrous acid-treated deoxyribonucleic acid. Second, because Lepesant et al. (5) have mapped a locus involved in the utilization of sucrose (*sacQ*) in this region of the chromosome, the possibility now exists that the gap between *pur*B linkage group and *sacQ* may be linked through *dal*. Finally, this requirement for D-alanine has permitted an efficient method for labeling peptidoglycan and teichoic acid for studies on the coordinate control of the biosynthesis of the cell wall in *B. subtilis* (M. J. Dul, F. E. Young, and A. N. Chatterjee, manuscript in preparation).

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