NOTES

Mapping of the Gene for a Major Penicillin-Binding Protein to a Genetically Conserved Region of the *Bacillus subtilis* Chromosome and Conservation of the Protein among Related Species of *Bacillus*

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Penicillin-binding protein 5 is the most abundant penicillin-binding protein in the vegetative membranes of *Bacillus subtilis* and accounts for 95% of the D,D-carboxypeptidase activity of the cell. The structural gene for penicillin-binding protein 5 was mapped to a genetically conserved region near *guaB* at 0° on the *B. subtilis* chromosome, and immunoassays revealed that there is conservation of this major penicillin-binding protein among related species.

Penicillin-binding protein (PBP) 5 accounts for at least 70% of the penicillin bound by the vegetative membranes of *Bacillus subtilis* (4, 28). It is the most abundant of the six vegetative PBPs and the smallest, with a calculated molecular mass of 45,494 daltons (29). At least 95% of the D,D-carboxypeptidase activity in *B. subtilis* can be attributed to this PBP, but the protein is not essential for vegetative growth (3). The cells survive the complete inactivation of *dacA*, the structural gene for PBP 5, and there is no detectable change in the degree of cross-linking of the vegetative peptidoglycan (27, 29). However, cells that lack PBP 5 do not form normal spores. The 10-fold reduction in heat resistance of the PBP 5-deficient spores implies a role for this major vegetative protein in formation of the cortex layer during sporulation (29).

Genetic mapping. Although there is no selectable phenotype associated with the absence of the *dacA* gene product, it was possible to map the gene by taking advantage of a chloramphenicol resistance marker (*cat*⁺; 17, 29) that had been inserted either within the gene or adjacent to it (strain JT175 or JT2000, respectively; Table 1). Mapping was done initially by transduction with bacteriophage PBS1 (12, 23), and the results were virtually the same with phage grown on either of these chloramphenicol-resistant (Cm^r) strains. Antibiotic resistance was determined on antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) containing 5 µg of either chloramphenicol or novobiocin (Nv) per ml. It was found that *dacA* (Cm^r) was 96 to 99% cotransducible with *guaB* (Table 2 and Fig. 1). Subsequent transformational crosses (1) confirmed this very close linkage. The *dacA* (Cm^r) gene was 75% cotransferred with *guaB* (150 Gua⁺/200 Cm^r), 13.6% cotransferred with *gyrB* (6 Nv^s/44 Cm^r), and 4.5% cotransferred with *div-355* (9 Ts⁻/200 Cm^r). This is the first of the PBP genes to be mapped in *B. subtilis*.

Interspecific studies of PBP 5. The chromosomal region around *guaB*, which maps near 0° on the 360° genetic map of *B. subtilis* (24), is highly conserved among a number of

TABLE 1. Strains of *B. subtilis* used for mapping *dacA*

Strain	Genotype	Source or reference
JT175	<i>trpC2 dacA::cat</i> ⁺	D. Ellar (29)
JT2000	<i>trpC2 dacA</i> ⁺ :: <i>cat</i> ⁺	D. Ellar (29)
NIG1154	<i>met his div-355</i>	Y. Sadaie (19, 26)
1A3	<i>cysA14 purA16 trpC2</i>	BGSC ^a
1A160	<i>gyrB (novA1) sacA321</i>	BGSC
1A291	<i>leuA8 metB5 purA16 tms-26</i>	BGSC
1A293	<i>guaB3 metC7 purH1 trpC2</i>	BGSC

^a BGSC, *Bacillus* Genetic Stock Center.

species of *Bacillus* and contains primarily genes involved in such essential and complex growth processes as protein synthesis, cell division, and sporulation (9, 10, 13). It is intriguing that *dacA* was located in this region, and it is

TABLE 2. Transductional mapping of the *dacA* gene

Recipient strain	Selected phenotype (no.)	Unselected phenotype (no.)
<i>div-355</i> (Ts)	Cm ^r (86)	Ts ⁺ (81) Ts ⁻ (5)
<i>guaB</i>	Cm ^r (184) Gua ⁺ (167)	Gua ⁺ (183) Gua ⁻ (1) Cm ^r (160) Cm ^s (7)
<i>gyrB</i> (Nv ^r)	Cm ^r (94)	Nv ^s (89) Nv ^r (5)
<i>purA cysA</i>	Cm ^r (147)	Pur ⁻ Cys ⁻ (39) Pur ⁺ Cys ⁻ (54) Pur ⁻ Cys ⁺ (35) Pur ⁺ Cys ⁺ (19)
<i>purA tms-26</i> (Ts)	Cm ^r (149)	Pur ⁻ Ts ⁻ (5) Pur ⁺ Ts ⁻ (11) Pur ⁻ Ts ⁺ (57) Pur ⁺ Ts ⁺ (76)

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FIG. 1. Genetic map of the *guaB* region of the *B. subtilis* chromosome (11) indicating the most probable position of *dacA*. The numbers between markers equal 100 minus the percent cotransduction of the markers. The arrows point to the unselected markers. Each number is the average of data from at least two crosses. The *div-355* and *tms-26* loci are enclosed by parentheses to emphasize the uncertainty of their relative positions (17, 19–21).

reasonable to predict that the *dacA* gene itself is genetically conserved. Its putative role in development of a fully heat-resistant spore qualifies it as an essential gene for the organism in its natural habitat. Moreover, some important phenotypic properties of PBP 5 are conserved (14); there is a vegetative protein similar to this PBP in size, relative abundance, and activity in other species of *Bacillus* (Fig. 2A). That there are indeed structural homologies among these proteins was confirmed by enzyme-linked immunoassays on Western blots (30) of membranes from seven different species of *Bacillus* (Fig. 2B). Immune rabbit serum was raised against PBP 5 that had been partially purified by penicillin affinity chromatography (5). The anti-PBP 5 antibodies were blot affinity purified as described by Olmsted (22) before use as a reagent in these studies. The secondary antibody was goat anti-rabbit immunoglobulin G (Fc) conjugated to alkaline phosphatase (Promega Corp., Madison, Wis.). Anti-PBP 5 antibodies cross-reacted with the corresponding PBP from other members of the so-called *B. subtilis* group, which is defined as *Bacillus licheniformis*, *B. amyloquelificans*, *B. pumilus*, and *B. subtilis* (25) (*Bacillus* strains used are listed in Table 3). No other membrane proteins in any of the samples reacted with the antibody preparation, which confirms and extends an earlier report that anti-PBP 5 does not cross-react with any of the higher-molecular-weight PBPs (7). In addition, the antibodies did not react at all with membranes from the *B. subtilis* mutant JT175, which cannot synthesize PBP 5 (data not shown).

There was no detectable cross-reaction between anti-PBP 5 antibodies and the putative PBP 5 homolog from either *Bacillus megaterium* or *B. sphaericus*. This result was not

surprising in regard to *B. sphaericus*, which is taxonomically well removed from the *B. subtilis* group and has a chemically distinct vegetative peptidoglycan (18, 25). Analyses of membranes from two different strains of *B. megaterium*, strain Texas (data not shown) and the type strain (Fig. 2), gave the same negative result. This apparent absence of immunological relatedness between the major vegetative PBPs of *B. megaterium* and *B. subtilis* is consistent with at least one genetic study, which suggested that the region of the chromosome where *dacA* has now been mapped is less highly conserved between these two species than between members of the *B. subtilis* group (13). It remains possible that there is some homology between the two PBPs that simply could not be detected by this approach.

Given the negative results with *B. megaterium*, it was not expected that the anti-PBP 5 antibodies would cross-react with the corresponding PBP from the more distantly related *B. cereus*. Nevertheless, a positive reaction on immunoblots was obtained with membrane samples from two different strains of this organism, strain T (data not shown) and the type strain (Fig. 2). One possible explanation is that *B. cereus* acquired the *dacA* gene from *B. subtilis* after the two species evolved.

Although there is obviously immunological relatedness among the major PBPs from some species of *Bacillus*, we do not yet know the actual extent of the homology. Immunological cross-reactivity is reportedly only detectable with polyclonal serum when there is at least 70 to 80% homology in the primary structures of the proteins (15), but it could be that these species share a single immunodominant region or a few highly antigenic regions, which constitute only a small fraction of the whole protein (2). This latter possibility appears to be the best explanation for the cross-reactivity between antibody raised against PBP 1a of *Streptococcus pneumoniae* and PBP 2 of *Streptococcus pyogenes* (16).

FIG. 2. (A) Fluorograph of a sodium dodecyl sulfate-polyacrylamide gel containing membrane samples from seven different species of *Bacillus*. Only the major [^3H]penicillin-labeled PBP in each sample is shown. Preparation of the membranes and the PBP assay have been described previously (6, 8, 28). (B) Immunoblot of the same seven samples. Lanes: a and h, *B. subtilis* 168 *trp*; b, *B. cereus*; c, *B. licheniformis*; d, *B. megaterium*; e, *B. pumilus*; f, *B. sphaericus*; g, *B. amyloquelificans*.

TABLE 3. *Bacillus* species used for comparative studies

Organism	Collection code ^a
<i>B. amyloquelificans</i>	BGSC 10A1
<i>B. cereus</i> T	BGSC 6A1
<i>B. cereus</i> type strain	ATCC 14579
<i>B. licheniformis</i> type strain	ATCC 14580
<i>B. megaterium</i> Texas	BGSC 7A2
<i>B. megaterium</i> type strain	ATCC 14581
<i>B. pumilus</i> type strain	ATCC 7061
<i>B. sphaericus</i> type strain	ATCC 14577

^a BGSC, *Bacillus* Genetic Stock Center; ATCC, American Type Culture Collection.

Further studies along these lines will require assays with monoclonal antibodies and sequence data for the PBPs from species besides *B. subtilis*.

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ADDENDUM

Shortly after the genetic studies were begun, we learned about physical mapping data that indicated a 238-kb genomic fragment spanning the region between *spo0J* and *spo0H* very likely included *dacA* (24a).

REFERENCES

- Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**:741-746.
- Arnheim, N., E. M. Prager, and A. C. Wilson. 1969. Immunological prediction of sequence differences among proteins. *J. Biol. Chem.* **244**:2085-2094.
- Blumberg, P. M., and J. L. Strominger. 1971. Inactivation of D-alanine carboxypeptidase by penicillins and cephalosporins is not lethal in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **68**:2814-2817.
- Blumberg, P. M., and J. L. Strominger. 1972. Five penicillin-binding components occur in *Bacillus subtilis* membranes. *J. Biol. Chem.* **247**:8107-8113.
- Blumberg, P. M., and J. L. Strominger. 1974. Covalent affinity chromatography as a means of purifying the penicillin binding components from bacterial membranes. *Methods Enzymol.* **34**:401-405.
- Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-88.
- Buchanan, C. E., J. Hsia, and J. L. Strominger. 1977. Antibody to the D-alanine carboxypeptidase of *Bacillus subtilis* does not cross-react with other penicillin-binding proteins. *J. Bacteriol.* **131**:1008-1010.
- Buchanan, C. E., and J. L. Strominger. 1976. Altered penicillin-binding components in penicillin-resistant mutants of *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **73**:1816-1820.
- Chilton, M. D., and B. J. McCarthy. 1969. Genetic and base sequence homologies in bacilli. *Genetics* **62**:697-710.
- Copeland, J. C., and J. Marmur. 1968. Identification of conserved genetic functions in *Bacillus* by use of temperature-sensitive mutants. *Bacteriol. Rev.* **32**:302-312.
- Dean, D. H., and D. R. Zeigler. 1989. *Bacillus* Genetic Stock Center strains & data, 4th ed. The Ohio State University, Columbus.
- Dedonder, R. A., J.-A. Lepesant, J. Lepesant-Kejzlarova, 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.
- Dubnau, D., I. Smith, P. Morell, and J. Marmur. 1965. Gene conservation in *Bacillus* species. I. Conserved genetic and nucleic acid base sequence homologies. *Proc. Natl. Acad. Sci. USA* **54**:491-498.
- Frere, J.-M., and B. Joris. 1984. Penicillin-sensitive enzymes in peptidoglycan biosynthesis. *Crit. Rev. Microbiol.* **11**:299-396.
- Gasser, F., and C. Gasser. 1971. Immunological relationships among lactic dehydrogenases in the genera *Lactobacillus* and *Leuconostoc*. *J. Bacteriol.* **106**:113-125.
- Hakenbeck, R., H. Ellerbrok, S. Tornette, and I. van de Rijn. 1987. Common antigenic determinants of pneumococcal penicillin-binding protein (PBP) 1a and *Streptococcus pyogenes* PBP 2. *FEMS Microbiol. Lett.* **48**:171-174.
- Haldenwang, W. G., C. D. B. Banner, J. F. Ollington, R. Losick, J. A. Hoch, M. B. O'Connor, and A. L. Sonenshein. 1980. Mapping a cloned gene under sporulation control by insertion of a drug resistance marker into the *Bacillus subtilis* chromosome. *J. Bacteriol.* **142**:90-98.
- Hungerer, K. D., and D. J. Tipper. 1969. Cell wall polymers of *Bacillus sphaericus* 9602. I. Structure of the vegetative cell wall peptidoglycan. *Biochemistry* **8**:3577-3587.
- Miyakawa, Y., and T. Komano. 1981. Study on the cell cycle of *Bacillus subtilis* using temperature-sensitive mutants. I. Isolation and genetic analysis of the mutants defective in septum formation. *Mol. Gen. Genet.* **181**:207-214.
- Nilsson, D., and B. Hove-Jensen. 1987. Phosphoribosyl-pyrophosphate synthetase of *Bacillus subtilis*. *Gene* **53**:247-255.
- Nilsson, D., B. Hove-Jensen, and K. Arnvig. 1989. Primary structure of the *tms* and *prs* genes of *Bacillus subtilis*. *Mol. Gen. Genet.* **218**:565-571.
- Olmsted, J. B. 1986. Analysis of cytoskeletal structures using blot-purified monospecific antibodies. *Methods Enzymol.* **134**:467-472.
- Pai, C. H. 1975. Genetics of biotin biosynthesis in *Bacillus subtilis*. *J. Bacteriol.* **121**:1-8.
- Piggot, P. J. 1989. Revised genetic map of *Bacillus subtilis* 168, p. 1-41. In I. Smith, R. A. Slepecky, and P. Setlow (ed.), *Regulation of procaryotic development*. American Society for Microbiology, Washington, D.C.
- 24a. Piggot, P. J., and M. Amjad. Unpublished data.
- Priest, F. G., M. Goodfellow, and C. Todd. 1988. A numerical classification of the genus *Bacillus*. *J. Gen. Microbiol.* **134**:1847-1882.
- Sadaie, Y., and T. Kada. 1983. Effect of septum-initiation mutations on sporulation and competent cell formation in *Bacillus subtilis*. *Mol. Gen. Genet.* **190**:176-178.
- Sharpe, A., P. M. Blumberg, and J. L. Strominger. 1974. D-Alanine carboxypeptidase and cell wall cross-linking in *Bacillus subtilis*. *J. Bacteriol.* **117**:926-927.
- Sowell, M. O., and C. E. Buchanan. 1983. Changes in penicillin-binding proteins during sporulation of *Bacillus subtilis*. *J. Bacteriol.* **153**:1331-1337.
- Todd, J. A., A. N. Roberts, K. Johnstone, P. J. Piggot, G. Winter, and D. J. Ellar. 1986. Reduced heat resistance of mutant spores after cloning and mutagenesis of the *Bacillus subtilis* gene encoding penicillin-binding protein 5. *J. Bacteriol.* **167**:257-264.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.