Isolation of *Bacillus subtilis* transformation-deficient mutants and mapping of competence genes

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Summary

We have isolated and characterized 48 *Bacillus subtilis* competence-deficient mutants. The mutants, obtained by nitrosoguanidine mutagenesis or by insertional mutagenesis with transposon Tn917, had a reduced transformation frequency and a wild-type transduction frequency. The com mutations were mapped by PBS1 transduction and at least four new com genes have been identified. The mutants were also characterized for their capacity to bind and take up the transforming DNA.

1. Introduction

The genetic transformation of *Bacillus subtilis* requires a physiological state known as competence (Young & Spizizen, 1961) and it is a complex process involving the following sequence of events (Dubnau, 1985; Smith *et al.* 1981): (i) binding of transforming DNA to a competent cell; (ii) entry of bound DNA which is rendered single stranded by a nuclease activity; (iii) incorporation of the single-stranded DNA into the recipient chromosome, leading to the formation of heteroduplex DNA; and (iv) resolution of heteroduplex DNA and expression of the newly acquired information.

During the development of competence *B. subtilis* cells undergo a series of physiological changes: one of the major changes is a decrease in the rate of DNA and RNA synthesis and the appearance of new proteins (Dooley *et al.* 1971; Smith *et al.* 1981). Mutants impaired in competence development (com mutants) can not be transformed, because they are unable to bind and/or take up DNA.

Several com mutants have been identified and partially characterized (Fani *et al.* 1984; Hahn *et al.* 1987; Mulder & Venema, 1982; Smith *et al.* 1983) and products that appear to be competence specific have been detected (Barberio *et al.* 1985; Eisenstadt *et al.* 1975; Finn & Landman, 1985; Smith *et al.* 1985). Altogether, the number of loci mapped is at least thirteen, including those described here. All the mutants isolated were defective in uptake, and many of them also failed to bind transforming DNA (Fani *et al.* 1984; Hahn *et al.* 1987). However, since many proteins have been described to be synthesized during competence development (Barberio *et al.* 1985) many more com genes remain to be identified.

The *B. subtilis* com mutations obtained by chemical mutagenesis suffer from the disadvantage that the mutated gene is not marked so as to allow easy isolation; furthermore, their mapping is very laborious. One way to circumvent these problems is to use insertional mutagenesis. In *B. subtilis* this can be accomplished by the use of transposon Tn917 (Youngman *et al.* 1983) from *Streptococcus faecalis*.

In this paper we report data on the isolation and mapping of new competence mutations obtained both by chemical mutagenesis and insertional mutagenesis with transposon Tn917.

2. Materials and methods

(i) Bacterial strains, bacteriophage and plasmid

The *B. subtilis* parental strain used was PB3361 (*purB6, leu8, metB5, trpC2, hisA1, lys21, thr5*). Phage PBS1 was used for transduction and mapping experiments. Plasmid pTV1 (Youngman *et al.* 1983) was kindly supplied by P. J. Youngman.

(ii) Media

The media were the minimal medium of Davis & Mingioli (1950) and Penassay medium (PY) antibiotic medium no. 3 (Difco).
(iii) DNA preparation
Transforming DNA was extracted as described by Marmur (1961). Plasmid DNA was prepared by the method of Gryczan et al. (1978). DNA concentration was determined by the diphenylamine method (Dische, 1955).

(iv) Transformation and transduction
Preparation of B. subtilis competent cells and transformation in liquid medium were carried out as previously described (Fani et al. 1984).

Transformation on solid medium was performed by streaking bacterial cells on PY medium and growing them at 37 °C; after 20 h, the streaks were replicated onto selective minimal agar spread with transforming DNA (50 µg per plate). Transduction experiments with PBS1 phage were performed by the method of Hoch et al. (1967).

(v) Selection and scoring of genetic markers
Selection for biochemical markers was performed on selective minimal agar. Plasmid-carrying strains resistant to chloramphenicol (Cm') were selected on PY medium containing 5 µg/ml Cm. The phenotype for resistance to macrolide, lincosamide and streptogramin B antibiotics (MLS'), conferred by the erm gene of Tn917, is inducible by erythromycin (Em). Induction was carried out in liquid medium containing 0.15 µg/ml Em; after 2 h of incubation at 37 °C, cells were plated on selective medium containing 1 µg/ml Em and 25 µg/ml lincomycin (Lm).

(vi) Isolation of competence mutants by insertional mutagenesis
Plasmid pTV1, containing transposon Tn917 and a temperature-sensitive origin of DNA replication, was transferred by transformation into the B. subtilis strain PB3361. For the mutagenesis experiments the strain PB3361 (pTV1) was grown overnight at 30 °C in PY broth containing Cm, Em and Lm at the above concentrations (after induction with Em). In the morning the cells were diluted 50-fold in the same medium and grown at 30 °C. When the culture had reached an OD,0.600, = 0.6-0.7, the cells were plated on PY agar containing Em and Lm at selective concentrations. Plates were incubated 48 h at 49 °C. At this temperature, plasmid pTV1 does not replicate and the only cells able to grow are the ones in which Tn917 has transposed into the chromosome.

Colonies grown on PY agar were screened on minimal medium containing Em (1 µg/ml) and Lm (25 µg/ml). After 48 h incubation at 49 °C, the colonies were scored for the isolation of transformation-deficient mutants by transformation on solid medium.

(vii) Total association and entry of transforming DNA
Total association and entry of transforming DNA in competent cultures were determined as described by Mulder & Venema (1982).

(viii) Mapping
The linkage relationship between the com mutations and biochemical markers of known location was determined by PBS1 transduction using the kit strains of Dedonder et al. (1977). The transduction mapping experiments were done at least twice and for each unselected marker more than 200 transductants were scored.

Map distances were expressed as

\[
(1 - \text{contransfer index}) \times 100
\]

(Nester & Lederberg, 1961). The contransfer index \( r \) is a measure of the frequency of joint transfer of two markers compared to the total number of recombinant genotypes measured by the transduction experiments. In a general notation, giving the genotypes \( a^1b^1 \) to the donor, \( a^0b^0 \) to the recipient, and the genotypes \( a^1b^1 \), \( a^0b^0 \) and \( a^0b^1 \) to the transductants:

\[
r = \frac{a^0b^1}{a^1b^1 + a^0b^0 + a^0b^1}.
\]

When it is possible to estimate \( a^1b^1 \) and \( a^0b^0 \) but not \( a^0b^1 \), we may assume \( a^0b^1 = a^1b^0 \).

3. Results

(i) Isolation of competence-deficient mutants
Competence mutants were isolated by insertional mutagenesis using the transposon Tn917 carried by the plasmid pTV1 (Youngman et al. 1983), which contains also a cat gene (resistance to Cm) and a B. subtilis temperature-sensitive origin of DNA replication. On PY agar 4100 colonies were obtained (the transposition frequency was about \( 10^{-5} \)). To eliminate new induced auxotrophic mutations, the bacteria were transferred to minimal medium containing the nutritional requirements of PB3361 and the antibiotics (1 µg/ml Em and 25 µg/ml Lm). The 2573 colonies (63%) which grew after this second selection were screened for their capacity to be transformed on solid medium. In this way 68 (1-7%) transformation-deficient strains were isolated and further characterized by measuring the transformation and transduction frequencies in liquid medium. The data obtained showed that 23 mutants had a reduced transformation frequency and a wild-type transduction frequency, therefore they were classified as com mutants; 13 mutants had reduced both transformation and transduction frequencies and were considered rec.

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mutants; the remaining 32 strains did not appear to be mutants.

On the basis of the transformation frequencies in liquid medium the 23 com mutants could be divided in two groups: one of 5 mutants with frequencies from $10^3$ to $10^4$ times lower than the parental strain and a second group of 18 mutants with frequencies from $10^4$ to $10^6$ times lower.

In a previous paper we described the isolation of 29 competence-deficient mutants obtained by nitrosoguanidine mutagenesis (Fani et al. 1984), which showed a strong reduction in transformation frequency ($10^3$–$10^5$ times less than the parental strain; data not shown). Four of these mutants have been characterized for other properties (Barberio et al. 1985; Fani et al. 1984); here we will report data concerning the previously uncharacterized mutants.

(ii) Mapping of the com mutations

The com mutations were mapped by PBS1 transduction, as described above. First, mutations obtained by nitrosoguanidine mutagenesis were mapped, and the Com$^-$ phenotype was scored by transformation on solid medium. This procedure sometimes gives ambiguous results, because it can happen that too few cells are transferred by replica plating. Therefore we were able to map only the mutations in 10 strains. Of the 10 mutations, one, designed com31 and carried by FB108, was located in a new locus, which maps between aro and dal (Fig. 1). Map distances are given from aro only, because neither the Com$^-$ nor the Dal$^-$ phenotypes were selectable in transduction. The order of the markers is supported by the fact that, 82% of the times that dal was co-transduced with aro, com31 was also. Furthermore, co-transduction was not observed between com31 and cysA, which is to the left of aro. The other nine mutations were distributed among the four com genes already described by Fani et al. (1984).

The mapping of the 23 mutations obtained with Tn917 was less laborious, because we could score the Com$^-$ phenotype by selecting for Em and Lm resistance. Fourteen mutations mapped between purB6 and tre12; only the map position of com14, carried by strain FB-T14, is shown (Fig. 2a). In our experiments the map distance between purB6 and tre12 is much larger than that published by Lepesant-Kejzarová et al. (1975), this is probably due to the presence of the Tn917 insertion which may lower co-transduction frequencies. We did not determine whether the other 13 mutations were located in the same gene or in a neighbouring one.

Mutation com18, carried by strain FB-T18, did not co-transduce with purB6 and was mapped between tre12 and glyB (Fig. 2b), as were two others. Three other mutations, which appeared to be not linked to tre12, mapped between glyB and metC, as shown for com44 of strain FB-T44 (Fig. 2c).

(iii) Binding and entry of transforming DNA

All the mutants were tested for their capacity to bind and take up transforming DNA; here we report data for the mutants described in Figs. 1–3. All the mutants mapping in the same position behaved similarly.

Strain FB108 (com31) is strongly reduced in
transformation ($10^3$ times less than the parental strain) and it does not bind transforming DNA (data not shown). The data on the other mutants (Table 1) show that they have also lost the capacity to bind transforming DNA. Note that the mutants FB-T67 and FB-T114, which were able to bind and take up DNA, although less efficiently than the parental strain, were also less defective in transformation, as might be expected.

4. Discussion

We have mapped the com mutations of several B. subtilis competence-deficient mutants. Using mutants previously obtained by nitrosoguanidine mutagenesis (Fani et al. 1984), we identified a new competence gene (com31), whereas nine other mutations mapped in loci where com mutations had already been located. The linkage of com31 with the arol marker might indicate a connection with the gene coding for a competence specific nuclease (Vosman et al. 1987), but we do not know whether the latter is located to the left or to the right of the arol gene.

Because of the problems found with the mapping of com mutations obtained by chemical or physical mutagenesis, we made new mutants using the transposon Tn917. We obtained 23 com mutants; of these, fourteen, including com14, had the transposon inserted between purB and tre12. This represents a new competence mutation. Two new com genes have been identified by com18 and com114 mutations, while com44 and com67 mapped in the same area as the previously described com30 and com104 mutations, respectively (Fani et al. 1984).

The map position of com110 might be the same of that of the class VII mutation described by Hahn et al. (1987). However, our mutation is not co-transduced with the lys-1 marker (data not shown) and the colonies are not sticky as has been described for the class VII mutants.

It is interesting to note that com67 has the same map position as the comW4 mutation, which in FB94 strongly reduced transformation frequency and DNA uptake, but not DNA binding (Fani et al. 1984). This might suggest that the com gene(s) located between

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sacA and purA could be involved in the entry of the transforming DNA.

In conclusion, we can say that four new com genes have been mapped and perhaps a fifth one (comI10). All the data concur to give the idea that many genes are involved in competence development and that they are spread all over the chromosome. More work has to be done to find out the products of these genes.

References

Barberio, C., Coppolecchia, R., Mastromei, G. & Polisinelli, M. (1985). Competence proteins in Bacillus subtilis com mutants. Biochimica Biophysica Acta 842, 184–188.

Davis, B. D. & Mingioli, E. S. (1950). Mutants of Escherichia coli requiring methionine or vitamin B12. Journal of Bacteriology 60, 17–28.

Dedonder, R. A., Lepesant, J. A., Lepesant-Kejzlarova, J., Billault, A., Steinmetz, M. & Kunst, F. (1977). Construction of a kit of reference strains for rapid genetic mapping in Bacillus subtilis. Applied and Environmental Microbiology 33, 989–993.

Dische, Z. (1955). Color reactions of nucleic acids components. In The Nucleic Acid, vol. 1 (ed. E. Chargraff and J. N. Davison), pp. 285–304. Academic Press, Inc., New York.

Dooley, D. C., Hadden, C. T. & Nester, E. W. (1971). Macromolecular synthesis in Bacillus subtilis during development of the competent state. Journal of Bacteriology 108, 668–679.

Dubnau, D. (1982). Genetic transformation in Bacillus subtilis. In The Molecular Biology of the Bacilli, pp. 147–178. Academic Press, New York.

Eisenstadt, E., Lange, R. & Willecke, K. (1975). Competent Bacillus subtilis cultures synthesize a denatured DNA binding activity. Proceedings of the National Academy of Sciences, U.S.A. 72, 323–327.

Fani, R., Mastromei, G., Polisinelli, M. & Venema, G. (1984). Isolation and characterization of Bacillus subtilis mutants altered in competence. Journal of Bacteriology 157, 152–157.

Finn, C. W. Jr. & Landman, O. E. (1985). Competence related proteins in the supernatant of competent cells of Bacillus subtilis. Molecular and General Genetics 198, 329–335.

Gryczan, T. J., Contente, S. & Dubnau, D. (1978). Characterization of Staphylococcus aureus plasmids introduced by transformation into Bacillus subtilis. Journal of Bacteriology 134, 318–329.

Hahn, J., Albano, M. & Dubnau, D. (1987). Isolation and characterization of Tn917lac-generated competence mutants of Bacillus subtilis. Journal of Bacteriology 169, 3104–3109.

Hoch, F. A., Barot, M. & Anagnostopoulos, C. (1967). Transformation and transduction in recombination defective mutants of Bacillus subtilis. Journal of Bacteriology 93, 1925–1937.

Lepesant-Kejzlarová, J., Lepesant, J. A., Walle, J., Billault, A. & Dedonder, R. (1975). Revision of the linkage map of Bacillus subtilis 168: indications for circularity of the chromosome. Journal of Bacteriology 121, 823–834.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. Journal of Molecular Biology 3, 208–217.

Mulder, J. A. & Venema, G. (1982). Isolation and partial characterization of Bacillus subtilis mutants impaired in DNA entry. Journal of Bacteriology 150, 260–268.

Nester, E. & Lederberg, J. (1961). Linkage of genetic units of B. subtilis in DNA transformation. Proceedings of the National Academy of Sciences, U.S.A 47, 52–55.

Smith, H. O., Danner, D. B. & Deich, R. A. (1981). Genetic transformation. Annual Review of Biochemistry 50, 41–68.

Smith, H., de Vos, W. & Bron, S. (1983). Transformation in Bacillus subtilis: properties of DNA binding deficient mutants. Journal of Bacteriology 153, 12–20.

Smith, H., Wiersma, K., Venema, G. & Bron, S. (1985). Transformation in Bacillus subtilis: further characterization of a 75,000 dalton protein complex involved in binding and entry of donor DNA. Journal of Bacteriology 164, 201–206.

Vosman, B., Kooistra, J., Olijve, J. & Venema, G. (1987). Cloning in Escherichia coli of the gene specifying the DNA entry nuclease of Bacillus subtilis. Gene 52, 175–183.

Young, F. E. & Spizizen, J. (1961). Physiological and genetic factors affecting transformation of Bacillus subtilis. Journal of Bacteriology 81, 823–829.

Youngman, P. J., Perkins, J. B. & Losick, R. (1983). Genetic transposition and insertional mutagenesis in Bacillus subtilis with Streptococcus faecalis transposon Tn917. Proceedings of the National Academy of Sciences, U.S.A. 80, 2305–2309.

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