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

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(*Received 16 June 1988 and in revised form 4 January 1989*)

Summary

We have isolated and characterized 48 *Bacillus subtilis* competence-deficient mutants. The mutants, obtained by nitrososoguanidine 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).

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(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^r) were selected on PY medium containing 5 μg/ml Cm. The phenotype for resistance to macrolide, lincosamide and streptogramin B antibiotics (MLS^r), 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 solid 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 $OD_{590nm} = 0{\cdot}6$–$0{\cdot}7$, 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 transductans 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^1b^0 and a^0b^1 to the transductans:

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

When it is possible to estimate a^1b^1 and a^1b^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*

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 10^2 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 *aroI* and *dal* (Fig. 1). Map distances are given from *aroI* 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-transducted with *aroI*, *com31* was also. Furthermore, co-transduction was not observed between *com31* and *cysA*, which is to the left of *aroI*. The other nine mutations were distributed among the four *com* genes already described by Fani *et al.* (1984).

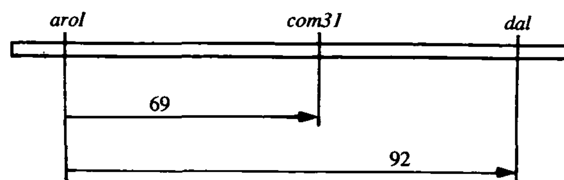
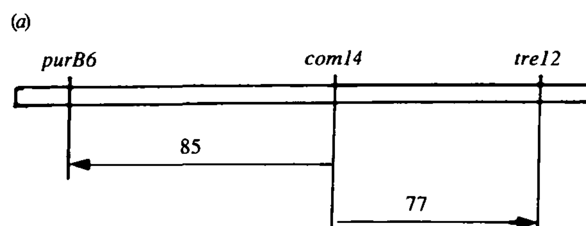
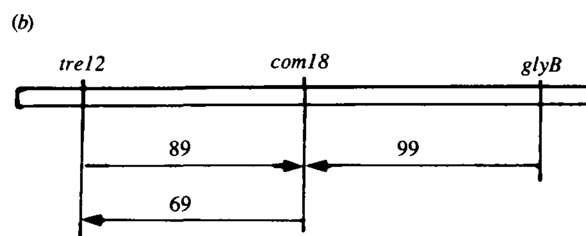


Fig. 1. Location of the *com31* mutation of strain FB108. The arrows point from the selected to the unselected marker.





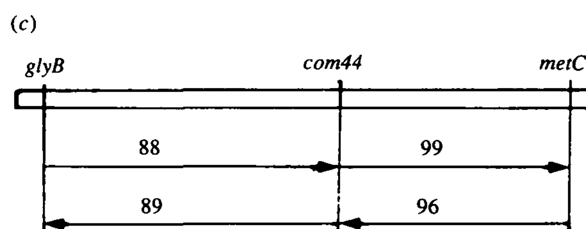


Fig. 2. Location of the *com14*, *com18* and *com44* mutations of the strains FB-T14 (*a*), FB-T18 (*b*) and FB-T44 (*c*). The arrows point from the selected to the unselected marker.

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. 2*a*). In our experiments the map distance between *purB6* and *tre12* is much larger than that published by Lepesant-Kejzlarovà *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. 2*b*), 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. 2*c*).

Mutations *com67* and *com110* mapped between *sacA* and *purA* (Fig. 3*a*) and between *aroD* and *leu* (Fig. 3*b*), respectively. Mutation *com114* was located between *pyrD* and *thyA* (Fig. 3*c*), because it was not co-transduced with *ilvA*.

(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.

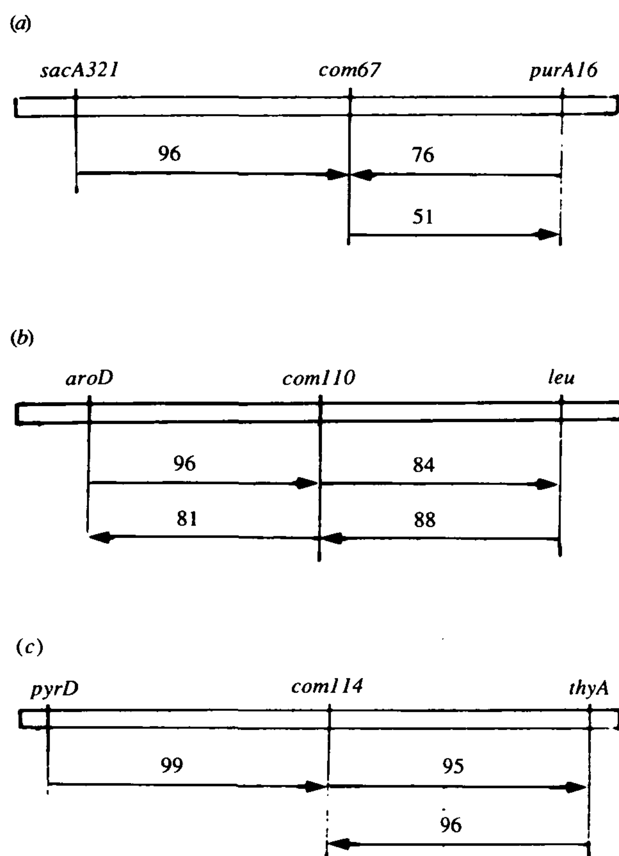


Fig. 3. Location of the *com67*, *com110* and *com114* mutations of the strains FB-T67 (*a*), FB-T110 (*b*) and FB-T114 (*c*). The arrows point from the selected to the unselected marker.

Table 1. *Binding and entry of transforming DNA*

Strain	Transformation Frequency[a]	^{3}H radioactivity (cpm per 10^8 cells): Total DNA association[b]	Entry[c]
PB3361 (pTV1)	$7{\cdot}5 \times 10^{-3}$	6586	3728
FB-T14	$3{\cdot}5 \times 10^{-7}$	75	32
FB-T18	$1{\cdot}0 \times 10^{-6}$	9	5
FB-T44	$1{\cdot}4 \times 10^{-6}$	9	14
FB-T67	$3{\cdot}7 \times 10^{-4}$	5823	2621
FB-T110	$3{\cdot}3 \times 10^{-8}$	78	7
FB-T114	$6{\cdot}8 \times 10^{-5}$	4056	2422

[a] Transforming ^{3}H-labelled *trp*$^+$ DNA (specific activity $1{\cdot}3 \times 10^5$ cpm/μg) was used at $1{\cdot}5$ μg/ml. Trp$^+$ transformed mutants were selected. Tranformation was for 30 min at 37 °C.

[b] Total radioactivity associated with cells, both sensitive and resistant to DNase I.

[c] Amount of DNase I resistant activity associated with the cells.

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 *aroI* 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 *aroI* 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 *com104* 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

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 (*com110*). 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.

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