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Linked Transformation of Bacterial and Prophage Markers in *Bacillus subtilis* 168 Lysogenic for Bacteriophage ϕ105

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Level of competence reached by *Bacillus subtilis* 168 lysogenic for temperate phage ϕ 105 was reduced compared to that reached by nonlysogenic cells. This effect was probably related to an alteration of the bacterial surface. Deoxyribonucleic acid extracted from ϕ 105 lysogenic bacteria was used to transform other lysogenic bacteria. About 25% linkage was found between the bacterial *phe-1* marker and prophage marker *ts* N15. The order of a few prophage markers relative to *phe-1* was established in three-factor crosses. The usefulness of this system for a study of the linkage between an integrated prophage genome and that of its host was discussed.

Temperate bacteriophage ϕ105 can lysogenize the transformable bacterium *Bacillus subtilis* 168. The gross genetic structure of vegetative and prophage ϕ105 has recently been described, and evidence has been presented that prophage and bacterial markers can reside on the same deoxyribonucleic acid (DNA) molecule active in transformation (7). Our experiments were undertaken with the aim of investigating transformation of bacteria lysogenic for phage ϕ105. Such a system should prove useful for a study employing cotransformation of bacterial and prophage markers as a measure of the linkage between the integrated prophage genome and that of its host.

MATERIALS AND METHODS

Bacteria and phage. *B. subtilis* 168, its derivative BR95 (*trp-2, phe-1, ilvA-1*) and their lysogenic derivatives, phage ϕ105 and the mutants of this phage employed, and phage PBS1 have all been described (7). Strain GSY 1025 (*trp-2, met-4, rec*$_1^-$), strain GSY 1026 (*trp-2, met-4*), and phage SP10 were provided by Hoch (3). BR95 (ϕ105) was cured of its prophage by transducing the cells with PBS1 prepared on nonlysogenic 168 and selecting for *phe*$^+$ and *ilv*$^+$ double transductants. About 1% of these transductants produced no phage and were sensitive to phage ϕ105.

Media and assay conditions. Media employed and the methods for growth and assay of phage and bacteria were described (7).

DNA preparation. Bacteria were grown in Penassay Broth (Difco) at 37 C on a shaker to a density of 2-4 $\times$ 10^8. The cells were centrifuged and suspended in about 2.5% volume of buffer [0.01 M tris(hydroxymethyl)-aminomethane (Tris), 0.02 M ethylenediaminetetraacetic acid (EDTA), and 3% sodium citrate (*p*H 8.1)]. Sodium dodecylsulfate was added to a final concentration of 0.25%, and the cells were incubated at 37 C for 15 to 30 min. The suspension was made 1 M with respect to NaCl and shaken with an equal volume of buffer-saturated phenol (Mallinckkrodt Chemical Works, St. Louis, Mo., reagent grade) at 4 C for 15 min. The two phases were separated by centrifugation and the aqueous phase was dialyzed against two changes of buffer [0.15 M NaCl, 0.015 M sodium citrate, 0.002 M EDTA, and 0.01 M Tris (*p*H 7.7)]. DNA concentrations were determined with the diphenylamine method of Burton.

Transformation procedure. Bacteria were grown for competence and bacterial and prophage recombinants were scored as described (7). DNA was pipetted with pipettes without tips in order to avoid shearing of the DNA which might destroy linkage between distantly linked markers (5).

RESULTS

Effect of prophage ϕ105 on the development of competence in B. subtilis. The level of competence reached by *B. subtilis* (ϕ105) is low compared to nonlysogenic bacteria (Table 1). This effect has been found for all markers tested in lysogenic strains. When a lysogenic bacterium is cured of the prophage it can again be transformed with the same high efficiency as the original nonlysogenic strain. Without success, we have tried to attain higher levels of competence in lysogenic cells by altering the time of incubation in growth medium

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TABLE 1. *Transformation of nonlysogenic and φ105 lysogenic strains*

Strain[a]	Marker selected	Percentage of transformants[b]
1026	*trp-2*	0.18
1026 (φ 105)	*trp-2*	0.009
1025	*trp-2*	0.04
1025 (φ 105)	*trp-2*	0.001
1026	*met-4*	0.38
1026 (φ 105)	*met-4*	0.007
BR95	*trp-2*	0.16
BR95 (φ 105)	*trp-2*	0.002
BR95C	*trp-2*	0.59
BR95	*phe-1*	0.40
BR95 (φ 105 N15)	*phe-1*	0.01
BR95 (φ 105 N10)	*phe-1*	0.01
BR95	*ilvA-1*	0.10
BR95 (φ 105 N15)	*ilvA-1*	0.009
BR95 (φ 105 N10)	*ilvA-1*	0.007

[a] Strains 1026 and 1025 were transformed with DNA from 168. BR95 was transformed with DNA from 168 (φ 105). About 0.1 μg of DNA per ml was added in each experiment. BR95C = BR95 (φ 105) cured of its prophage.

[b] Percentage of transformants was calculated by comparison with the number of colonies appearing on TBAB plates.

or transformation medium. It is thus important to investigate whether the prophage has a direct effect on the transforming DNA such as through the appearance of a nuclease activity which will destroy added DNA, or whether lysogeny affects the cells such that they will take up or integrate DNA less readily.

There is no increased production of infectious phage by competent lysogenic cultures exposed to DNA. Preincubation of DNA with culture filtrates from competent lysogenic cells does not impair the ability of the DNA to transform other competent cells (Table 2). Lysogenic cells grown for competence do not excrete any stable substance which inhibits the development of competence; nor do nonlysogenic cells excrete a substance which will promote the development of competence in a lysogenic culture (Table 3).

From strain BR95 (φ105) colonies (200) transformed for *trp*$^+$ or *phe*$^+$ were examined for lysogeny; all of the transformants had retained the prophage. Thus, the transformed cells from a competent lysogenic culture do not represent a small fraction of cells which have lost the prophage (Tables 6 and 7).

Frequency of transfection of φ105 lysogenic bacteria with DNA from the virulent phage φ1 (6) is reduced to the same extent as the frequency of formation of *trp*$^+$ transformants (Table 4), whereas phage φ1 plates with equal efficiency on

TABLE 2. *Effect of culture filtrates of strains 1026 and 1026 (φ 105) on the transforming activity of DNA extracted from B. subtilis 168[a]*

Incubation medium	Percentage of *trp*$^+$ transformants
TM	0.44
S-1026	0.58
S-1026 (φ 105)	0.54

[a] Strains 1026 and 1026 (φ 105) were grown to competence. The bacteria were centrifuged and the supernatant fluid was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). DNA from 168 was incubated at a concentration of 1 μg per ml for 30 min at 37 C in transformation medium (TM), supernatant fluid from competent 1026 culture (S-1026), and supernatant fluid from competent 1026 (φ 105) culture (S-1026 φ 105).) The DNA solution (0.1 ml) was then added to a competent culture of 1026 and after 30 min incubation the bacteria were plated for *trp*$^+$ transformations and total colony-formers.

TABLE 3. *Development of competence in strains 1026 and 1026 (φ 105) incubated in culture filtrates from the same strains[a]*

Strain	Uptake medium	Minutes of incubation before addition of DNA	
		30	90
		% *trp*$^+$	% *trp*$^+$
1026	TM	0.02	0.46
	S-1026	0.02	0.23
	S-1026 (φ 105)	0.02	0.15
1026 (φ 105)	TM	0.0001	0.0008
	S-1026	0.0004	0.0006
	S-1026 (φ 105)	0.0002	0.0007

[a] Strains 1026 and 1026 (φ 105) were grown in growth medium 4.5 hours. They were then diluted into TM, S-1026, or S-1026 (φ 105). At the times indicated 0.1 μg of DNA from 168 was added and 30 min later the samples were assayed for *trp*$^+$ transformants and total colony-formers.

the two strains. On the other hand, lysogenic and nonlysogenic cells are transduced with equal efficiency by phages SP10 or PBS1 (Table 5). These experiments demonstrate that lysogenic bacteria can integrate a DNA fragment introduced into the cell as well as nonlysogenic ones (3) and indicate that only a small fraction of competent lysogenic cells can bind DNA. This latter suggestion is supported by the fact that the time of appearance and rate of increase of deoxyribonuclease-insensitive transformants are

TABLE 4. *Transformation and transfection of strains 1026 and 1026 (φ 105)*[a]

Strain	Percentage of *trp*+ transformants	Percentage of infectious centers
1026	0.06	0.16
1026 (φ 105)	0.0015	0.005

[a] To competent cultures of the two strains was added 1 μg of bacterial DNA or 10 μg of φ 1 DNA. After 30 min incubation, *trp*+ transformants were scored. After 45 min incubation, infectious centers were scored by plating on 168 (φ 105) indicator bacteria.

TABLE 5. *Transduction of lysogenic and nonlysogenic strains with SP10 and PBS1*[a]

Transducing phage	Strain	Selected marker	Transductants per ml
SP10-1	1026	*trp-2*	8,700
SP10-2	1026	*trp-2*	7,800
SP10-1	1026 (φ 105)	*trp-2*	5,800
SP10-2	1026 (φ 105)	*trp-2*	15,100
PBS1	BR95	*phe-1*	520
PBS1	BR95	*ilvA-1*	570
PBS1	BR95 (φ 105 N15)	*phe-1*	860
PBS1	BR95 (φ 105 N15)	*ilvA-1*	1,110

[a] The SP10-transducing lysates were prepared as described by Hoch, Barat, and Anagnostopoulos (3). PBS1 transducing lysates were prepared on 168 (φ 105).

the same in lysogenic and nonlysogenic cultures, whereas the total number of transformants formed is quite different.

Some poorly transformable derivatives of *B. subtilis* had an increased resistance to actinomycin D (2). Nonlysogenic and φ105-lysogenic *B. subtilis*, however, showed no difference in their sensitivity to this drug.

Transformation of prophage markers. Experiments established that the low level of competence reached by lysogenic cells is due neither to degradation of added DNA outside the cell nor to an impaired ability to integrate DNA taken up by the cells. Rather, prophage φ105 seems to affect the entry of DNA into the cells. Linkage between prophage and bacterial markers was then investigated.

Prophage φ105 has been shown to be linearly integrated into the bacterial chromosome between the *phe-1* and *ilvA-1* markers (7). BR95 lysogenic for φ105 carrying one of the temperature-sensitive (*ts*) mutations N15, N22, N10, or N31 was used as recipient. The order of the markers employed, *phe-1*, N15, N22, N10, N31, *ilv-1*, was established by PBS1 transduction (7).

DNA extracted from 168 (φ105) was added to competent cultures of BR95 (φ105*ts*) and *phe*+ and *ilv*+ tranformants were selected. The frequency of cotransfer of a particular prophage marker was scored by transferring the colonies to plates seeded with indicator bacteria and incubating at 30 or 40 C (7). These experiments showed that N15 and N22 are closest to *phe-1* but did not show a definitely higher cotransfer of N10 or N31 with either of the bacterial markers (Table 6).

Prophage marker N15 was the one most frequently cotransformed with a bacterial marker (*phe-1*). Five different DNA preparations were examined for this linkage. Among 150 *phe*+ transformants analyzed for each sample, 25 to 30% cotransfer of N15 was found. Cotransformation of *phe-1* and N15 should be a useful indicator of the integrated versus the nonintegrated state of the φ105 genome. Preliminary experiments employing DNA extracted at intervals after prophage induction of 168 (φ105) showed at least a fivefold decrease in *phe-1*, N15 cotransfer at the time of initiation of phage DNA synthesis, whereas the *trp-2 his-2* linkage was unaffected.

Finally, a set of three-factor crosses was performed to establish the relative order of the above prophage and bacterial markers. In these crosses, DNA from 168 lysogenic for various *ts* mutants of φ105 was used to tranform BR95 lysogenic for other *ts*mutants. The presence of recombinant prophage was scored among the *phe*+ and *ilv*+ recombinants. The results of these experiments confirm the order *phe-1*, N15, N22 (Table 7). The order of N10 and N31 relative to the bacterial markers cannot be established by transformation, however. Although both of these markers are cotransformed with both *phe-1* and *ilvA-1* at a significant frequency, the frequency of recom-

TABLE 6. *Cotransformation frequencies between bacterial and prophage markers*[a]

Prophage marker	Selected bacterial markers		
	phe-1	*ilvA-1*	*trp-2*
N15	44/208	12/208	2/208
N22	29/208	6/208	2/156
N10	36/460	31/416	0/190
N31	38/464	31/416	2/256

[a] About 0.5 μg of DNA extracted from 168 (φ 105) per ml was added to competent BR95, lysogenic for the *ts*-mutants indicated. Values indicate ratios of transformed colonies yielding phage plating at 40 C to total colonies analyzed.

TABLE 7. *Three-factor crosses between prophage and bacterial markers*[a]

Prophage markers		Selected bacterial marker		
Donor	Recipient	*phe-1*	*ilvA1*	*trp-2*
N22	N15	45/520	2/520	0/988
N15	N22	0/163	0/129	0/359
N31	N10	8/416	4/312	
N10	N31	27/520	19/520	

[a] To competent BR95 lysogenic for *ts* mutants indicated (recipient) was added about 0.5 μg of DNA from 168 lysogenic for other *ts* mutants (donor). Values indicate the ratios of colonies yielding phage plating at 40 C to total colonies.

binant prophages is the same irrespective of the bacterial marker selected.

DISCUSSION

The level of competence reached by *B. subtilis* (φ105) is considerably reduced compared to non-lysogenic cells. This effect depends on the presence of the prophage and thus constitutes an example of lysogenic conversion. Several cases of lysogenic conversion have been described previously in other *Bacillus* species (4; Jonasson, Rutberg and Young, *in preparation*). The presence of the prophage does not affect the integrity of extracellular DNA nor does it impair the ability of the bacteria to integrate DNA introduced into the cell. Our results are most compatible with the idea that the prophage causes an alteration of the surface of the bacteria such that the fraction of cells capable of taking in DNA is reduced.

About 25% cotransformation was found between bacterial marker *phe-1* and prophage *ts*-marker N15. By employing the frequency of cotransfer of these two markers in transformation with DNA extracted from lysogenic bacteria it should be possible to study in vitro the nature of the linkage between the prophage and host genomes. A purified fragment of DNA, carrying both the bacterial and prophage markers, might also prove suitable as a substrate for identification of the enzyme(s) which promotes integration and excision of the prophage. Three-point tests by transformation confirmed the order of *phe-1*, N15, and N22 previously suggested by PBS1 transduction (7) and further demonstrated that the order of these markers on the prophage genetic map is congruent with the position of the respective mutations on the DNA molecule. Although prophage markers N10 and N31 are cotransferred with *phe-1* or *ilv-1* at a significant frequency, the results do not allow us to determine the relative order of these markers. The results may suggest that markers brought into the cell on the same DNA molecule, but sufficiently apart, are integrated into the bacterial chromosome independently of each other. The minimal length of such a distance is at least 2×10^7 double-stranded molecular weight, since this is roughly the distance between N15 and N31 as calculated (8) from previously published data on PBS1 transduction of prophage markers (7), and assuming the size of the PBS1 transducing fragment to be about 1.5×10^8 molecular weight (1).

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