RecE-dependent Lysogenic Induction in the Absence of Repressor in *Bacillus subtilis* Non-complementing Diploids

By NANCY GUILLÉN, AHMED ZAHRAOUI, RICHARD D'ARI AND LUISA HIRSCHEIN

1Institut de Microbiologie (Bât. 409), Université Paris-Sud, 91405 Orsay Cedex, France
2Institut Jacques Monod, CNRS, Université Paris 7, 2 Place Jussieu, Paris, 75251 Paris Cedex 05, France

(Received 12 November 1985; revised 25 January 1986)

The RecE protein of *Bacillus subtilis*, known to be required for induction of the SOS response and of $\phi$105 prophage, was shown to be involved in mitomycin C induction of *B. subtilis* diploid lysogens carrying a silent $\phi$105 prophage in their unexpressed chromosome. These stable non-complementing diploid lysogens, formed by protoplast fusion and regeneration, did not synthesize repressor, so that the induction observed must have resulted from RecE-dependent activation of the prophage rather than from RecE-dependent inactivation of repressor. Mitomycin C treatment does not induce permanent expression of the silent chromosome, so the activation seems to be temporary, perhaps reflecting the action of an SOS function under RecE control.

INTRODUCTION

As first reported by Hotchkiss & Gabor (1980), fusion of *Bacillus subtilis* protoplasts of two genetically marked strains can produce a class of heterozygous diploid bacteria which carry the two parental chromosomes but express only one of them. These bacteria, called non-complementing diploids (Ncds), have the phenotype of one parent but segregate cells of the other parental phenotype at frequencies of $10^{-2}$ to $10^{-4}$. We have subsequently described a new class of stable Ncds, which segregate cells of the unexpressed parental phenotype at a frequency of $10^{-7}$ (Guillén et al., 1985).

Unstable Ncds may be an intermediate in the formation of stable Ncds. In unstable Ncds the unexpressed chromosome seems to form a distinct nucleoid structure. The transforming activity of this DNA, detected by selecting for unexpressed markers, is very low in crude lysates but is restored to normal by treatment with proteinase K or by DNA purification (Guillén et al., 1982a; Bohin et al., 1982). The DNA from stable Ncds, on the other hand, has low transforming activity for unexpressed markers even after purification, suggesting that inactivation of the chromosome results from modifications of the DNA itself rather than from its tertiary structure (Guillén et al., 1985).

In Ncd strains in which a $\phi$105 prophage was present in the expressed chromosome, $\phi$105-specific mRNA was synthesized; but when the prophage was in the unexpressed chromosome, no $\phi$105 specific mRNA was detected (Guillén et al., 1982b). Furthermore, the latter Ncds were not immune to $\phi$105 superinfection and were not induced at high temperature when the prophage carried a thermolabile repressor mutation. Thus the inactivation of the unexpressed chromosome in Ncds is sufficiently complete to maintain a prophage in the absence of repressor.

Non-immune Ncd lysogens are induced to produce phage by mitomycin C treatment (Guillén et al., 1982b). In *Escherichia coli*, lysogenic induction involves RecA-directed repressor cleavage associated with induction of the SOS response (Walker, 1984). In immune *B. subtilis* lysogens,

**Abbreviation:** Ncd, non-complementing diploid.
induction requires a functional RecE protein (Love & Yasbin, 1984), analogous to the E. coli RecA protein (de Vos et al., 1983), and probably involves a pathway of repressor inactivation similar to that in E. coli (Friedman & Yasbin, 1983). On the other hand, induction of a non-immune Ncd lysogen must require activation of prophage genes rather than repressor inactivation. Since mitomycin C reacts directly with DNA, producing interstrand crosslinks and bulky adducts (Iyer & Szybalski, 1963), it was possible that these modifications resulted directly in chromosome activation and prophage induction, in which case induction should not require RecE activity. We show here that mitomycin C induction of a φ105 prophage in the unexpressed chromosome of an Ncd does require active RecE protein, suggesting that induction – and perhaps chromosome activation – may result from the action of some SOS function.

**METHODS**

_Bacterial strains_. The _B. subtilis_ strains used are listed in Table 1. Those constructed for the present work were obtained by lysogenization with φ105 wild-type phage (Rutberg, 1969).

_Media_. For fusion experiments, bacteria were cultured in nutrient broth (Schaeffer et al., 1965) and protoplasts reverted to bacillary form in a rich regeneration agar medium (R medium) of high tonicity (Wyrick et al., 1973). For Ncd selection, minimal medium was used (Anagnostopoulos & Spizizen, 1961), supplemented with growth factors as needed.

_Fusion and subculturing of Ncd strains_. Fusion experiments were done according to Schaeffer et al. (1976). Modifications were introduced in the proportion of protoplasts used in recE+ x recE4 crosses (1:100). After isolation, Ncd strains were subcloned and stabilized by growth in minimal selective medium; their diploidy was checked by self-fusion experiments (Guillén et al., 1985). Stable Ncd clones were conserved in 20% (v/v) glycerol at −70°C.

_Prophage induction and phage assay_. Nutrient broth cultures of Ncd lysogens were induced by adding 1 μg mitomycin C ml⁻¹ for 10 min. The cells were then centrifuged, resuspended in nutrient broth and incubated for 20 min at 37°C. Infective centres were assayed by plating appropriate dilutions with GB43 indicator bacteria on nutrient broth plates, using a 3 ml soft agar overlay. The plates were incubated for 24 h at 37°C before counting.

**RESULTS**

_Product of fusion crosses between recE+ and recE4 strains_

To test the RecE dependence of mitomycin C induction of non-immune Ncd lysogens, we had to construct, by fusion crosses, appropriate Ncd strains carrying the recE4 mutation and a φ105 prophage. With recE+ strains, as previously reported (Hotchkiss & Gabor, 1980; Schaeffer et al., 1976), about 10% of the protoplasts regenerate bacillary forms, and 5% of these clones are unstable Ncds. With recE4 strains, the regeneration frequency was 100-fold lower, and 2% of the clones were unstable Ncds (Table 2). In fact, the recE4 mutants are hypersensitive to the process of protoplast formation; the regeneration frequency was equally low in the absence of polyethylene glycol treatment. To obtain heterozygous recE+/recE4 Ncds, we found that a ratio of 1 recE+ to 100 recE4 protoplasts gave the best results: 1% to 2% of the regenerated clones were unstable Ncds (Table 2). Curiously, about 1.8% of the clones presented recombinant phenotypes (non-parental auxotrophs); these presumptive recombinants were not characterized further.

_Role of the RecE protein in induction of Ncd lysogens_

From the above fusion crosses, stable Ncds of the desired genotypes were isolated. We verified that the Ncd lysogens with the prophage in the unexpressed chromosome were not immune to φ105, whereas those with the prophage in the expressed chromosome were immune.

To evaluate the inducibility of these strains, we measured the fraction of bacteria that produced an infective centre when plated on a sensitive lawn before or after treatment with mitomycin C. As expected, all strains with a recE+ gene in the expressed chromosome were inducible. When the expressed chromosome carried the recE4 mutation, however, no induction was observed, whether the prophage was on the expressed or the unexpressed chromosome (Table 3). Thus induction of non-immune Ncd lysogens by mitomycin C shows the same recE+ dependence as induction of immune lysogens or of the SOS response.
Table 1. Bacillus subtilis strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7</td>
<td>purB34 leu-8 metB5 rfm-486</td>
<td>Schaeffer et al. (1976)</td>
</tr>
<tr>
<td>S15</td>
<td>purB34 ura-1 trpC7 rfm-486</td>
<td>Anagnostopoulos &amp; Spizizen (1961)</td>
</tr>
<tr>
<td>GB43</td>
<td>thr-3 leu-8 metB5 sup-3</td>
<td>Sanchez-Rivas &amp; Garro (1979)</td>
</tr>
<tr>
<td>BD224</td>
<td>thr-3 trpC2 recE4</td>
<td>Dubnau et al. (1973)</td>
</tr>
<tr>
<td>GSY1277</td>
<td>metC3 recE4 Leu- Ery-</td>
<td>C. Anagnostopoulos, Gif-sur-Yvette, France</td>
</tr>
<tr>
<td>S1(φ105)</td>
<td>thr-5 leu-8 metB5 rfm-486 (φ105)</td>
<td>Schaeffer et al. (1976)</td>
</tr>
<tr>
<td>S7(φ105)</td>
<td>purB34 leu-8 metB5 rfm-486 (φ105)</td>
<td>This work</td>
</tr>
<tr>
<td>BD224(φ105)</td>
<td>thr-5 trpC2 recE4 (φ105)</td>
<td>This work</td>
</tr>
</tbody>
</table>

Table 2. Fusion products of recE+ and recE4 strains

Fusion crosses were done as indicated in Methods and followed by regeneration on R medium. The colonies were replicated onto selective media for phenotypic classification. Cell wall regeneration frequency is strain dependent; for the other frequency results the values were identical in two determinations.

<table>
<thead>
<tr>
<th>Cross</th>
<th>No. of cell wall regenerated bacteria analysed in each cross</th>
<th>Frequency of Ncd clones</th>
<th>Frequency of recombinant phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>S15 × S1(φ105)</td>
<td>10⁻¹</td>
<td>5.7 × 10⁻²</td>
<td>≤3 × 10⁻³</td>
</tr>
<tr>
<td>GSY1277 × BD224(φ105)</td>
<td>1.5 × 10⁻³</td>
<td>2 × 10⁻²</td>
<td>≤3 × 10⁻³</td>
</tr>
<tr>
<td>S7 × BD224(φ105)</td>
<td>4 × 10⁻³</td>
<td>2 × 10⁻²</td>
<td>1.6 × 10⁻²</td>
</tr>
<tr>
<td>BD224 × S7(φ105)</td>
<td>5 × 10⁻³</td>
<td>1 × 10⁻²</td>
<td>2 × 10⁻²</td>
</tr>
</tbody>
</table>

Table 3. recE dependence of induction of non-immune Ncd lysogens

Viable cells and segregants were determined by plating appropriate dilutions of Ncd cultures on rich and selective media. Mitomycin C treatment and infective centre assays were done as described in Methods; the values are means of three plates, and the results agreed to within 0.1%.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Segregation of non-expressed phenotype</th>
<th>Infective centres/total bacteria†</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1(φ105)</td>
<td>Rec⁺(φ)</td>
<td>Without mitomycin C With mitomycin C</td>
</tr>
<tr>
<td>BD224(φ105)</td>
<td>Rec⁻(φ)</td>
<td>2 × 10⁻³ 0.8</td>
</tr>
<tr>
<td>S15 [S1(φ105)]</td>
<td>Rec⁺ [Rec⁺(φ)]</td>
<td>8 × 10⁻⁵ 7 × 10⁻⁵</td>
</tr>
<tr>
<td>S1(φ105) [S15]</td>
<td>Rec⁺ [Rec⁺(φ)]</td>
<td>1 × 10⁻² 0.52</td>
</tr>
<tr>
<td>GSY1277 [BD224(φ105)]</td>
<td>Rec⁻ [Rec⁻(φ)]</td>
<td>3 × 10⁻³ 0.8</td>
</tr>
<tr>
<td>BD224(φ105) [GY1277]</td>
<td>Rec⁻ [Rec⁻(φ)]</td>
<td>2 × 10⁻⁴ 2 × 10⁻⁴</td>
</tr>
<tr>
<td>S7 [BD224(φ105)]</td>
<td>Rec⁺ [Rec⁺(φ)]</td>
<td>4 × 10⁻⁵ 2 × 10⁻⁵</td>
</tr>
<tr>
<td>BD224 [S7(φ105)]</td>
<td>Rec⁻ [Rec⁻(φ)]</td>
<td>5 × 10⁻² 0.3</td>
</tr>
</tbody>
</table>

* The non-expressed chromosome or phenotype is shown in square brackets.
† Bacteria were assayed without mitomycin C treatment.

RecE protein is not required for prophage excision or development: when a haploid recE4 strain lysogenic for a φ105 mutant encoding a thermosensitive repressor was shifted to non-permissive temperature, 100% of the cells were induced and produced phage. Furthermore, mitomycin C treatment of the same recE4 thermostable lysogen did not affect its capacity to propagate φ105: 80% of the bacteria remained capable of producing phage at non-permissive temperature.

Non-immune Ncd lysogens have a higher rate of spontaneous induction than immune lysogens. Both spontaneous and mitomycin C induction require a functional recE⁺ gene on the expressed chromosome. The presence of a recE⁺ gene on the unexpressed chromosome cannot replace this requirement, confirming the inactivation of the recE gene (Table 3).

To see whether mitomycin C treatment of Ncds caused permanent activation of the entire unexpressed chromosome, we measured its effect on the frequency of segregation of the
unexpressed phenotype in non-lysogenic Ncds. No significant increase was found (data not shown), suggesting that chromosome activation by mitomycin C is incomplete or temporary.

**DISCUSSION**

Lysogenic bacteria, discovered by Lwoff *et al.* (1950), harbour a dormant prophage which is passed on to progeny cells as a stable part of the bacterial genome. Temperate bacteriophage can by definition establish a state of lysogeny, and the prophage makes its host immune to superinfection by the same phage. Subsequent work has established that the dormant prophage state and the superinfection immunity are both due to the presence of a prophage-encoded repressor which directly or indirectly prevents transcription of most of the prophage genes (Ptashne, 1971).

We have recently described a striking exception to this model of lysogeny, *Ncd* non-immune lysogens of *B. subtilis* (Guillén *et al.*, 1982b). The strains are true lysogens in that the prophage is stably transmitted to progeny, occasional cells produce phage spontaneously and immune segregants can be obtained by selecting for markers on the unexpressed chromosome. Prophage maintenance in Ncd non-immune lysogens must depend on the mechanism that maintains one chromosome in an inactive state.

The formation and maintenance of Ncds is an intriguing problem. We have previously used non-immune Ncd lysogens to show that the unexpressed chromosome does not direct the transcription of detectable mRNA, at least from the prophage (Guillén *et al.*, 1982b). One is thus led to imagine that the unexpressed chromosome is somehow unavailable to RNA polymerase. Furthermore, this barrier to transcription seems to reside in the DNA itself rather than in its cellular location or conformation, since purified DNA from stable Ncds has very low transforming activity for markers on the unexpressed chromosome (Guillén *et al.*, 1985).

The prophage in non-immune Ncd lysogens can be induced by treatment with the DNA-damaging drug mitomycin C (Guillén *et al.*, 1982b). It seemed possible that certain types of DNA lesions could activate the unexpressed chromosome. For example, structures created by stalled replication forks or by DNA repair processes might temporarily relieve the transcriptional barrier.

In the present work we show that mitomycin C induction of non-immune Ncd lysogens requires a functional recE+ gene on the expressed chromosome. Thus prophage activation is not the direct result of DNA lesions in the unexpressed chromosome.

The RecE protein is necessary for intermolecular recombination in *B. subtilis* (Dubnau *et al.*, 1973), and it is possible that mitomycin C treatment stimulates such events, resulting in prophage activation by recombination with the expressed chromosome. This hypothesis seems unlikely, however, because mitomycin C induces the prophage efficiently (30% to 50% of the cells), yet it does not stimulate the formation of segregants — or recombinants — of the unexpressed parental phenotype.

This last observation suggests that mitomycin induced activation reflects a temporary change in the DNA structure. In the case of a φ105 prophage, this activation is irreversible, resulting in lytic development and formation of an infective centre. For other chromosomal genes this putative activation must be reversible, since the original inactive state is ultimately restored.

A transient RecE-dependent effect induced by mitomycin C could well reflect the action of an inducible SOS function. *B. subtilis* is known to possess DNA repair and mutagenic activities inducible by DNA-damaging treatments, and this induction requires a functional RecE protein (Love & Yasbin, 1984). Induction of non-immune Ncd lysogens may require an SOS function affecting transcriptional capacity or, more likely, DNA structure to permit transcription of part or all of the unexpressed chromosome. We are currently trying to define the functions involved in mitomycin C induced activation of the unexpressed chromosome of *B. subtilis* Ncd strains.

We wish to thank Olivier Huisman for suggesting a possible involvement of the SOS response in prophage activation by mitomycin C. A. Z. was recipient of a fellowship from the Direction des Relations et de la Coopération Internationales of the Centre National de la Recherche Scientifique. This work was supported by
grants from the Centre National de la Recherche Scientifique (LA136, ATP Microbiologie) and the Fondation pour la Recherche Médicale Française.

REFERENCES


