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Stabilized non-complementing diploids (Ncd) from fused protoplast products of *B. subtilis*

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Non-complementing diploids (Ncd) displaying the parental phenotype can be selected from polyethylene glycol (PEG)-treated fused polyauxotrophic protoplasts of *Bacillus subtilis*. These bacteria carry the two parental genomes, but only one of them is phenotypically expressed, the other being replicated but not expressed. Cellular cloning and DNA-DNA *in situ* hybridization led to the discovery of non-complementing diploid cells which at first sight could have been considered as parental haploids. The new class of stabilized Ncd (10^{-7} segregants) can be obtained either directly after the primary fusion event or from segregating Ncd after further growth. The totally inactive chromosome of a stable Ncd can be activated after PEG-induced self fusion. DNA-mediated transformation studies using crude stable Ncd lysates as DNA donors show low frequencies for the genetic markers from the 'silent' chromosome. Contrary to the unstable Ncd situation, however, these frequencies remain low even with purified donor DNA. The differences in the transformation properties of the non-expressed markers are correlated to Ncd clone stability. These facts suggest that chromosome inactivation in PEG-induced fusion involves at least a two-stage process. The first would be reversible and the second irreversible, thus preserving the inactive chromosome state.

Key words: stable diploids/chromosome extinction/transformation/cellular cloning/chromosome reactivation

Introduction

Protoplast fusion in two parental *Bacillus subtilis* strains, each one carrying different multiple auxotrophic genetic markers, produces prototrophic diploid colonies: Cd (Lévi *et al.*, 1977; Lévi-Meyrueis *et al.*, 1980), and auxotrophic or prototrophic recombinant cells (Scheffer *et al.*, 1976; Gábor and Hotchkiss, 1979). In addition to these classes a new unexpected fusion product is also obtained. The latter comprises colonies that, while showing the phenotype of either of the original strains used in the fusion (to be called parental), segregates different classes of recombinants or colonies with the phenotype of either parent (Hotchkiss and Gábor, 1980). It necessarily follows that these fusion products contain both parental chromosomes of which one is reversibly inactivated. These classes of fusion products which, when derived from a single colony after repeated purifications, have been named non-complementing diploids (Ncd).

An inactivated chromosome can persist in this state for serveral generations (Hotchkiss and Gábor, 1980). The non-expressed genome has a much more reduced transformation activity (Guillén *et al.*, 1982; Bohin *et al.*, 1982). Previous studies have also shown that the non-expressed chromosome is not transcribed (Guillén *et al.*, 1983). As expected, an Ncd in which one of the chromosomes carries a ϕ105 prophage does not show immunity if the phage is inserted in the silent chromosome. Furthermore, studies to be published elswhere show that there is no difference in the methylation pattern of either 5′ CCGG or 5′ GATC between the active and inactive chromosome. However, studies concerning the mechanism of chromosome inactivation in *B. subtilis* would be easier if diploids with a stably inactivated chromosome were available.

Published data on the subject furnishes an interesting and apparent contradiction; while the proportion of fusion events involving the different parental types was found to be between 25 and 50% by two independent methods (Sanchez-Rivas and Garro, 1979; Frehel *et al.*, 1979), the estimated complementing diploids (Lévi-Meyrueis *et al.*, 1980), recombinants and unstable Ncd (Hotchkiss and Gábor, 1980), recovered from a non-selective regeneration medium, constituted $<10\%$ of the fusion events. This suggests that the remaining majority of heterologous fusion products must belong to a new, as yet undescribed class, one which includes stable Ncd.

Here we identify such a class in clones originating from fusion products that also yield unstable Ncd. The presence of an inactive chromosome is revealed in the putative stable Ncd by *in situ* hybridization and secondary reactivation promoted by a further fusion event.

Results

Analysis of fusion products

Hotchkiss and Gábor (1980) call biparental clones those colonies, produced after a fusion event, which when replicated from a non-selective regeneration medium are able to grown on each of the media supplemented with the required factors for each parent, while not being able to grow on a minimal medium. This operational definition does not prejudge the nature of these biparental colonies (B.P.). Furthermore, unstable Ncd will behave as biparental in the test described above. In fact, unstable Ncd are a subclass of the larger class of biparental clones (Sanchez-Rivas *et al.*, 1982; Gábor and Hotchkiss, 1983). They display a parental phenotype and are capable of segregating the other parental phenotype. The latter will only grow on a medium supplemented with its auxotrophic requirements.

Recovery of B.P. clones and parental phenotype of unstable Ncd

After a S15 x S1ϕ fusion cross, 1000 colonies from the regeneration medium were analyzed. About 6% of these clones (primary clones) had a B.P. phenotype according to the criteria described above (Hotchkiss and Gábor, 1980). Thus they grow in both parental media. The primary B.P. clones were further characterized by replicating each one with toothpicks on a minimal medium and a minimal medium supplemented with either parental growth factors. Table II shows that out of 61 primary B.P. clones, 22 are unstable Ncd with the S15 parental phenotype, 19 are unstable Ncd with the S1ϕ parental phenotype, while 20 belong to a new class of Ncd. Unlike the classical unstable Ncd, in which the segregants behave like a haploid cell, the segregants in this new class continue, over generations, to show a B.P. phenotype. The nature of this class remains unclear and was not

Table I. Bacterial strains

Strains used	Genotype	References
For fusion		
S15	*purB-34, trpC7, ura-1, rfm-486*	Levi *et al.*, 1977
S1ϕ	*metB-5, leu-8, thr-5, rfm-486* ϕ105	Schaeffer *et al.*, 1976
As recipients in transformation		
S8	*purB-34, thr-5, leu-8, rfm-486*	Schaeffer *et al.*, 1976
S6	*thr-5, ura-1, trpC7, rfm-486*	Schaeffer *et al.*, 1976
S9	*metB-5, ura-1, trpC7, rfm-486*	Schaeffer *et al.*, 1976
As indicator of ϕ105		
GB43	*metB-5, leu-8, thr-5, sup*$^{+3}$	Sanchez-Rivas and Garro, 1979

Table II. Analysis of primary B.P. clones by growth on minimal and parental selective media

Clones B.P. analyzed: 61	From MM + AUθ			From MM + MLT			Unstable Ncd phenotype	% of each phenotype
	Growing in AUθ	MLT	MM	Growing in AUθ	MLT	MM		
22/61	+	+	−	−	+	−	S15(S1ϕ)[a]	36
19/61	+	−	−	+	+	−	S1ϕ(S15)[a]	31
20/61	+	+	−	+	+	−	S15(S1ϕ)[a] + S1ϕ(S15)[a]	33

[a]In parentheses the non-expressed chromosome: strain S15 = $A^-U^-\theta^-Rif^R$; strain S1ϕ = $M^-L^-T^-Rif^R$ lysogenized with ϕ105 phage.
Abbreviations: M, methionine; θ, tryptophan: U, uracil; A, adenine; T, threonine; L, leucine; MM, minimum medium.

investigated further. When the unstable Ncd were grown in rich liquid medium, subcloned and replicated, most of the colonies lost the ability to segregate, thus showing a stable parental phenotype. This already suggests that stable non-complementing diploids might come from segregating Ncd.

Single colony analysis of fusion product clones from the regeneration medium. Among the 61 B.P. primary clones identified by their progeny as giving rise to only one of two possible types of unstable Ncd, six random colonies were suspended and plated, after appropriate dilution, on a variety of selective media. Colonies originating from single cells had heterogenous phenotypes in the B.P. clone (Table III). The majority had the two parental phenotypes with a lower number of biparental colonies. The relative proportion of these different classes varies widely among the six clones analyzed. In each case, one of the parental phenotypes constitutes the majority class. Since putative stable non-complementing diploids coming directly from the non-selective regeneration plates or from unstable Ncd do not behave as biparental clones, we thought it reasonable to search for this new class among the cells composing the original biparental colonies which produce unstable Ncd and their progeny.

Identification of stable non-complementing diploids

Considering that the putative stable Ncd and parental haploid

Table III. Cellular cloning after cell wall regeneration and growth in isolated colonies in several minimal media

B.P. clone identified by the segregating Ncd phenotype	Independent colonies	Colonies growing in selective medium		
		Phenotype		
		S1ϕ105	S15	Biparental[b]
S15(S1ϕ)[a]	224	35	188	1
S15(S1ϕ)	400	390	6	4
S15(S1ϕ)	300	10	290	0
S15(S1ϕ)	100	59	40	1
S15(S1ϕ)	400	360	10	30
S1ϕ(S15)	200	4	196	0
Total	1624	858	730	36

[a]In parentheses the non-expressed chromosome: S1ϕ = $M^-L^-T^-Rif^R$ ϕ105; S15 = $A^-U^-\theta^-Rif^R$. Six primary B.P. clones with a previously identified segregation Ncd phenotype were picked from a cell wall-regeneration medium, diluted and plated in rich medium. Independent colonies were analyzed on minimal medium and minimum medium supplemented with the growth factors required for each parent.
[b]Showing growth in both the MM + AUθ and MM + MLT media but no growth in a minimal medium.

Table IV. Colonies carrying a specific DNA sequence inserted in a non-expressed chromosome

Origin of Ncd clone		Phenotype		
		S1ϕ105	S15	Biparental
Non-selective cell wall-regeneration medium	Total number of colonies	66	33	1
	Number of hybridizing colonies	66	33	1
Selective medium	Total number of colonies		100	
	Number of hybridizing colonies		100	

S1ϕ105 genotype: *metB5, leu8, thr5, rfm486,* ϕ105. S15 genotype: *purB34, ural, trpC7, rfm486.* Ncd clone: S15(S1ϕ105), S1ϕ105 chromosome is unexpressed in this clone. All Ncd clones were subcloned and isolated colonies were transferred to selective media in order to define their phenotype. Hybridization was performed according to Materials and method.

strains cannot be distinguished by a segregation test we used two alternative strategies to reveal the presence of a stably inactivated chromosome.

Physical identification. Clones containing a non-expressed chromosome can be distinguished from a phenotypically identical haploid strain if the inactive chromosome carries an additional sequence that could be revealed by a DNA-DNA hybridization technique. We engineered this difference by fusing non-lysogenic cells with cells lysogenic for phage ϕ105. Using the products of these fusions, we analyzed after plating for single cells, those primary B.P. clones classified as unstable Ncd of the type which do not express the marker in the chromosome carrying the prophage (as described above, and in Table II). Four clones were thus analyzed with substantially similar results. Out of 100 colonies isolated after resuspension of one of the primary B.P. clones growing on a non-selective medium, 33 have a parental phenotype identical to the non-lysogenic parental strain S15. However, these 33 colonies hybridize with a ϕ105 DNA probe (Table IV). Thus, stable Ncd are found among products from non-selective regeneration plates.

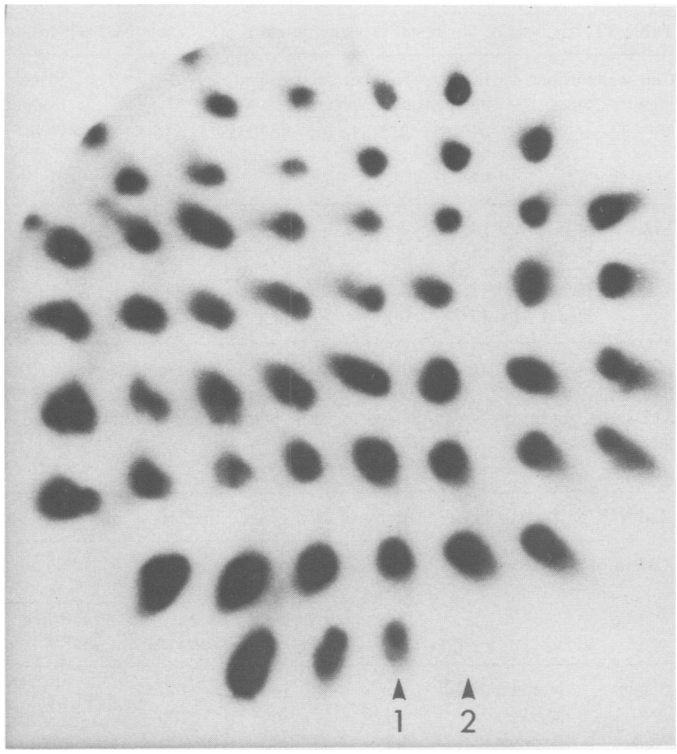

Fig. 1. *In situ* hybridization of stabilized S15(S1ϕ105) clones. The colonies were transferred to a nitrocellulose filter and grown overnight in contact with nutrient broth plates, treated with lysozyme. *In situ* hybridization was performed according to Grunstein and Hogness (1975) with [^{32}P]ϕ105 labelled DNA. Colonies from **(1)** lysogenic and **(2)** non-lysogenic parental haploid strains.

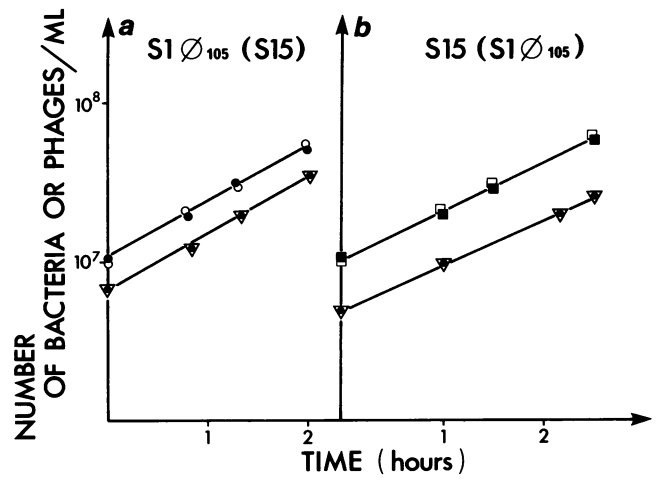

Fig. 2. Bacterial growth and infectious centers. Ncd clones were grown in a minimal medium supplemented with the growth factors of both parents. At different times during growth, aliquots of the culture were diluted, plated on selective media for both parents and their phenotype was determined. ϕ105 expression was measured during growth after mitomycin C induction by counting the infectious centers either on: **(a)** the expressed or **(b)** non-expressed chromosome in the Ncd cells. The segregation frequencies during growth were $<10^{-7}$ (see Materials and methods). Number of S1ϕ105(S15) or S15(S1ϕ105) rich media (● ■); minimum medium supplemented with MLT (○) or AUθ (□); number of p.f.u. (▲).

Furthermore, an unstable Ncd clone grown in a selective medium for five generations has its segregation capacity reduced from 10^{-3} to 10^{-5}. Studies of the same clone for 50 generations carried out as above suggest that the diploid state is stabilized since it has a 10^{-8} segregation frequency. An analysis of 100 of these subcloned cells shows that all of them have the same phenotype as the parental strain S15 (i.e., the chromosome carrying the prophage is not expressed) and hybridize with the ϕ105 DNA probe (Table IV, Figure 1). These results demonstrate that further growth of an unstable Ncd produces a stabilized Ncd clone, since the latter does not segregate while still containing the non-expressed sequences or at least some of them.

Presence of an intact phage genome in the non-expressed chromosome. We reported in a previous publication that a prophage inserted in the silent chromosome can be induced with mitomycin C (Guillén *et al.*, 1983). In this study we used this observation to detect the presence of a functionally intact phage genome in stabilized Ncd clones. Putative stable Ncd from reciprocal phenotypes were grown on a minimal medium supplemented with all the factors necessary for the growth of both parental types. Aliquots from these cultures were taken at various time intervals and tested for the segregation of the non-expressed chromosome and for the presence of a functional phage genome by measuring the number of infectious centers after mitomycin induction. Figure 2 (a,b) illustrates the correlation between the number of infectious centers and the total number of bacteria in the culture, regardless of which of the two possible unstable Ncd types they were derived from. In the S15 (S1ϕ) growth culture (Figure 2b), between 35 and 70% of the cell population carries the prophage, with an average of 55%. This ratio never exceeds 70% in either the S1ϕ (S15) (Figure 2a), or in the lysogenic parental haploid growing cells (not shown). Neither of the Ncd colonies segregates the non-expressed chromosome during their growth. If segregation occurs in the stabilized clones, the frequency will be $<10^{-8}$ compared with a frequency of 10^{-2} for unstable Ncd (Sanchez-Rivas *et al.*, 1982; Guillén *et al.*, 1983). Furthermore, DNA was extracted from cells harvested from both cultures and analyzed by Southern blotting after *Eco*RI enzyme digestion. DNA extracted from the vegetative phage ϕ105 was used as a probe.

The *Eco*RI restriction patterns of ϕ105 DNA from both Ncd cultures are identical (Figure 3). These contain bands corresponding to 11.7 kb, 9 kb, 7.6 kb, 5.3 kb, 3.2 kb, 1.5 kb, 0.86 kb and 0.48 kb fragments, as in the control lane containing DNA from the vegetative phage (Bugaichuk *et al.*, 1984), with an additional 2-kb fragment characteristic of ϕ105 DNA integrated in the bacterial chromosome (Guillén *et al.*, in preparation). Taken together, these results provide conclusive evidence that the entire prophage genome is contained in stabilized Ncd, regardless of whether it is inserted in the active or the inactive chromosome.

Reactivation of the non-expressed chromosome by self-fusion. The preceding experiments do not distinguish between a true diploid and a merozygote carrying only the region of chromosome with the prophage inserted in it. Therefore we attempted to reveal the entire non-expressed chromosome by a physiological rather than by a physical test. Since fusion events result in unstable Ncd (Hotchkiss and Gábor, 1980), we thought that a second fusion might destabilize stable Ncd clones, leading to the reactivation of all markers, if these were still present. Protoplasts were obtained from stable S15 (S1ϕ) Ncd and from haploid S15 cells. Two fusion crosses were then performed in the presence of polyethylene glycol (PEG): the first one was a self-fusion of the S15 (S1ϕ) Ncd and the other concerned S15 (ϕ) x S15. Prototrophic as well as B.P. clones were recovered among the regenerated

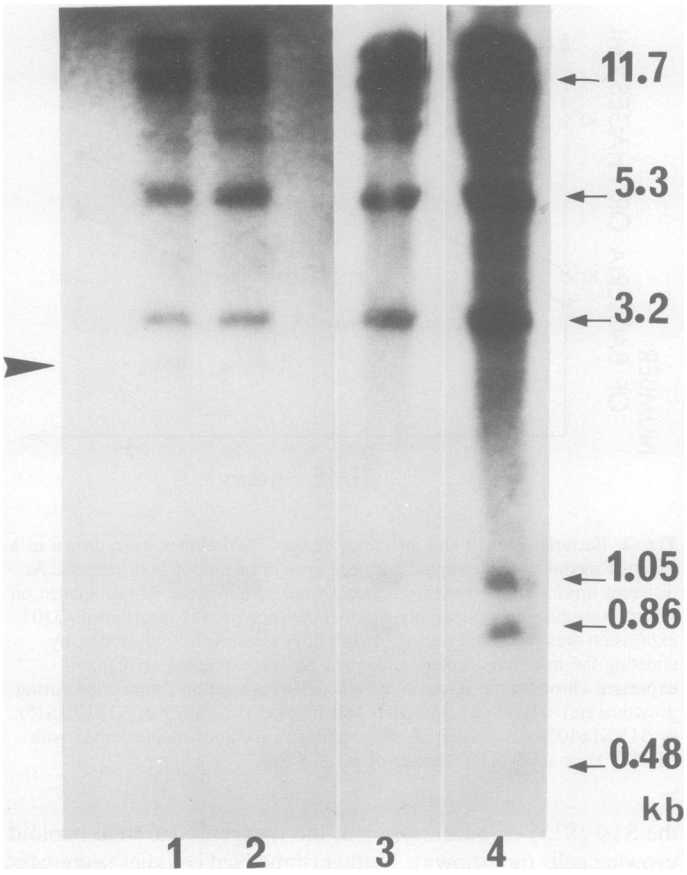

Fig. 3. Restriction endonuclease *Eco*RI cleavage pattern of 105 prophage DNA. Hybridization of radiolabelled ϕ105 DNA to Southern blot of *Eco*RI digest of genomic DNA S15(S1ϕ105) **(lane 1)**, S1ϕ105(S15) **(lane 2)**. Both were autoradiographed for 2 h and the ϕ105 phage DNA was autoradiographed for 2 h **(lane 3)** or for 24 h **(lane 4)**.

Table V. Reactivation of non-expressed chromosome after stable Ncd self-fusion

Strain	Regeneration medium	Number of colonies analyzed	% of phenotypes obtained among regenerated cells		
			Prototrophs	Biparental	Parental
Ncd	Selective	60	–	–	100
	Non-selective	150	–	–	100
Ncd + PEG	Selective	26	57.6	38.4	–
	Non-selective	411	–	3.5	96.5
Ncd + S15 + PEG	Selective	28	14	60.7	25
	Non-selective	258	–	4.2	95.8

Ncd strain: $A^-U^-\theta^-(M^-L^-T^-$ ϕ105); S15 strain: $A^-U^-\theta^-$. Fusion crosses as described in Materials and methods. After fusion the bacteria were grown in the indicated cell-wall regeneration medium and the colonies were tested in several selective media.

fusion product bacteria from both crosses (Table V). Conversely if the PEG treatment was omitted only parental types appeared.

The analysis of single randomly picked B.P. clones reveals (Table VI), segregating Ncd of both parental phenotypes, prototrophic recombinants and the new class of segregating Ncd, characterized by their ability to switch phenotypic expression. It follows that a fusion product which behaves like a stable parent can generate a B.P. clonal heterogeneity. This reinforces the original search for its diploid status.

Table VI. Analysis of biparental progeny obtained after stable Ncd self-fusion

Cell wall regeneration	Fusion cross	B.P. independent clones tested	Number of segregating Ncd clone phenotypes found			Prototrophic clones
			S15(S1ϕ105)	Sϕ105(S15)	S15(S1ϕ105) and S1ϕ105(S15)	
Non-selective	Ncd x Ncd	14	2	0	12	0
	Ncd x S15	11	7	0	2	2
Selective	Ncd x Ncd	11	0	0	11	0
	Ncd x S15	17	0	11	6	0

Ncd strain: S15(S1ϕ105); S1ϕ105 strain; $M^-L^-T^-$ lysogenized with ϕ105; S15 strain: $A^-U^-\theta^-$. B.P. clones from Ncd self-fusion and from a S15 x Ncd fusion cross were tested for their phenotype in several selective media.

Table VII. Transformation activity of Ncd genetic markers

Origin of Ncd clone			Origin of donor DNA (transformants per ml)		
			Lysate	Lysate + proteinase K	Purified DNA
Stable	Non-expressed	pur^+	140	140	140
	Markers	trp^+	60	200	200
Ncd	Expressed	leu^+	112 000	105 000	130 000
	Markers	thr^+	32 000	40 000	22 000
Unstable	Non-expressed	pur^+	600	13 800	–
	Markers	trp^+	1300	68 900	60 000
Ncd	Expressed	met^+	16 000	21 000	54 000
	Markers	thr^+	15 000	18 000	–

Transformation experiments were performed as described in Materials and methods. Phenotypes of both types of Ncd; $A^-U^-\theta^-(M^-L^-T^-)$; S8=$A^-L^-T^-$(1.4 x 10^8 bact./ml); recipient strains S6 = $U^-\theta^-T^-$(2 x 10^8bact./ml); S9 = $U^-M^-\theta^-$(2 x 10^8 bact./ml). Reversion frequencies: leu^+, thr^+, met^+: 10^{-8}; pur^+, trp^+: 10^{-7}.
pur = purine, adenine was used; trp = tryptophan; leu = leucine; thr = threonine; met = methionine.

Transformation activity

In a previous study concerning segregating Ncd, a correlation was found between non-expression *in vivo* and the loss of transformation activity *in vitro* when the transformation was performed with crude lysate (Bohin *et al.*, 1982). However, the lysate recovered the transformation activity for the non-expressed markers when treated with proteinase K (Bohin *et al.*, 1982) or when the DNA was otherwise purified (Hotchkiss and Gábor, 1980, and Table VII). These results suggest that the changes responsible for both inactivation and loss of transforming ability involve the structure of the nucleoid rather than affecting the DNA itself. This does not seem to be the case for stabilized Ncd. Table VII shows the transformation activity for expressed and non-expressed markers of a crude lysate, lysates digested by proteinase K and purified DNA, obtained from stable and unstable Ncd, respectively. Crude lysate obtained from a non-segregating Ncd shows a very low transformation activity for the non-expressed markers, as for the unstable class. However, this low activity is not increased by proteinase K digestion or by using purified DNA.

Discussion

This study has established that PEG-induced fusion of polyauxotrophic *B. subtilis* protoplasts can produce stable non-complemen-

ting diploids (stNcd) despite the initial assumption that these were the parental haploid strains (Schaeffer *et al.*, 1976; Hotchkiss and Gábor, 1983; Schaeffer and Hirschbein, 1985).

The Ncd described by Hotchkiss and Gábor (1980) had been identified by their capacity to segregate the non-expressed parental phenotype, therefore by definition they were segregating Ncd (seNcd). We found, however, that after regeneration on a non-selective medium, most of the clones identified as seNcd are in fact a mixture, in which the above non-complementing diploids are a minority. Most of the colonies had a stable parental phenotype (28%), Table III, thus confirming previous observations (Hotchkiss and Gábor, 1983). This result could be expected if the commitment to chromosome inactivation takes place either during or soon after bacterial protoplast reversion to the bacterial form (Hotchkiss and Gábor, 1985).

When seNcd are grown under selective conditions they lose the ability to segregate. All the progency again have a parental phenotype (Table IV). Stabilization of seNcd seems to be clonal dependent. Some seNcd are stabilized after a few generations of growth while others require at least 50 generations. Since the preceding results show that a putative stNcd containing both parental chromosomes might be generated either from the primary fused clone or from seNcd progeny, the presence of a whole inactive chromosome was investigated in both cases.

Using fusion crosses between a lysogenic and ϕ105 sensitive strain we obtained a seNcd with an expressed chromosome which did not carry the prophage. Once the seNcd was stabilized by further growth on a selective medium and using the prophage as indicator of the retention of the non-expressed chromosome, we found that 55% of the stabilized cell population contained the prophage, as estimated by the number of infectious centers. Since the lysogenic parental haploid strain gave 70% of infectious centers, this figure could be even more significant.

This result is further supported by the *in situ* hybridization experiments. When hybridized with labelled ϕ105 DNA, 100% of the subcloned randomly isolated colonies, coming from the regeneration plates or the seNcd progeny, with a phenotype identical to the non-lysogenic parental haploid, contain the same phage sequence.

The DNA extracted from these cells shows the same ϕ105 DNA restriction fragments as DNA extracted from the progeny of a prophage-carrying seNcd in the active chromosome.

These results lead to the conclusion that the stable diploid possesses a complete ϕ105 genome inserted in the inactive chromosome.

It can be argued that the existence of an entire chromosome cannot be proven by the presence of one of its markers. We therefore performed self-fusion experiments using cells with a stable parental phenotype, taking into account the fact that the fusion process induces variability in clonal stability (Gábor and Hotchkiss, 1983; Hotchkiss and Gábor, 1983). Protoplasts from stable Ncd were fused in the presence of PEG, and the regenerated cells were analyzed. Consistent with our prediction, we obtained segregating Ncd and prototrophic colonies, clearly indicating the diploidy of these cells.

It is worth noting that most of the colonies from a primary clone, identified as unstable Ncd, have a parental phenotype and are stable diploid cells. However, quantitative data on the abundance of the latter remain to be obtained, since this would require the analysis of a much larger number of clones than those examined in the present work. Nevertheless, protoplast fusion can produce very stable diploids which when properly investigated reveal the extent of gene non-expression.

Unlike the seNcd situation (Hotchkiss and Gábor, 1980), DNA-mediated transformation studies, with purified DNA from stabilized clones as a donor, show low transformation frequencies for the non-expressed markers. A similar observation was reported when the properties of a stable prototrophic diploid recombinant were studied (Lévi-Meyrueis and Sanchez-Rivas, 1984). An antibiotic sensitive clone, when used in transformation experiments, produced a low but significant number of drug-resistant transformants. Thus, testing of DNA by transformation might be a simple way to define gene contents in the clonal population of a stable diploid. However, since the transformation activity of the non-expressed markers is very low, this test will never eliminate the trivial possibility that we are dealing with a mixture which contains one parent in very small proportions. Cell population cloning and self-fusion experiments on stable diploid will then unambiguously reveal an entirely non-expressed chromosome. On the other hand, the unexpected finding that purified donor DNA, extracted from stable and unstable Ncd has strikingly different transformation activities, raises the interesting possibility that the mechanism underlying chromosome inactivation involves at least a two-step process as postulated for the mammalian somatic X chromosome (Gartler and Riggs, 1983).

In bacterial diploids, which are able to segregate, proteins associated with the inactive chromosome conceal the transformation activity of its markers (Bohin *et al.*, 1982; Le Hégarat *et al.*, 1982). On the contrary, the lack of transformation ability reactivation is correlated with Ncd stabilization (this work and Lévi-Meyrueis and Sanchez-Rivas, 1984). The two inactivation stages might be related with conformational changes in the nucleoids (Hirschbein and Guillén, 1982). The first step would be reversible and the second irreversible, thus ensuring the maintenance of the inactive state.

This prediction can be tested. Indeed, the existence of single or multiple specific DNA sequences on which the inactivation process operates can be proven by isolating mutants mapping in such regions. If these sequences are necessary to maintain the irreversible inactive state of the chromosome, such mutations could be obtained by the transposon mutagenesis (Youngman *et al.*, 1983) of a stable Ncd, followed by the selection of the semi-stable class. These experiments are in progress.

Materials and methods

Chemicals and enzymes

Mitomycin C and deoxyribonuclease I were purchased from Sigma Chemical Co., St. Louis, USA. DNA polymerase I and *Eco*RI endonuclease were from Boehringer, Mannheim, FRG. Labelled deoxycytidine 5′ [α-^{32}P]triphosphate (635 Ci/mmol) was from Amersham International, UK.

Bacterial and bacteriophage strains

B. subtilis strains used in the present experiments are listed in Table I. The phage strain used is wild-type ϕ105.

Media and buffer

Parental and Ncd strains were grown in nutrient broth (Schaeffer *et al.*, 1965). For segregation studies Ncd clones were grown in a minimal liquid medium (Anagnostopoulos and Spizizen, 1961), supplemented with the necessary growth factors.

Bacteriophage stocks were obtained using L.B.M. medium (Rutberg *et al.*, 1968). To count the p.f.u. of ϕ105 phage, R12 medium was used (Sanchez-Rivas and Garro, 1979).

ϕ105 phage production

Prophage induction, phage assay and ϕ105 purification were performed according to Guillén *et al.* (1983).

B. subtilis DNA extraction

DNA was isolated from Ncd clones or from parental strains in exponentially growing cells as described by Saunders *et al.* (1984).

Fusion experiments and Ncd selection

Bacterial protoplast fusions were performed according to Schaeffer *et al.* (1976). Selection of non-complementing diploids clones was carried out as described by Hotchkiss and Gábor (1980).

The plates containing the primary regenerated protoplast colonies can be conserved for 1 week at 4°C. Colonies identified as Ncd clones either from a cell-wall regeneration medium or from a selective one were preserved in rich medium containing 15% glycerol at −70°C.

Cloning of a cell population and single colony analysis

Clones classified as Ncd or presumtive Ncd were resuspended in 1 ml of nutrient broth, diluted, plated on complete medium and grown overnight at 37°C. Well-isolated colonies from plates containing no more than 10 clones were picked, transferred on several diagnostic media and analyzed for further classification.

Self-fusion procedure

A phenotypic parental diploid, detected as stable Ncd, was grown in rich liquid medium for several generations. Protoplast fusion and regeneration procedures were performed as described by Schaeffer *et al.* (1976) and Sanchez-Rivas (1982).

Genetic transformation

Transformation was carried out by adding as donor DNA either 0.1 ml of fresh Ncd lysate prepared according to Bohin *et al.* (1982), or 0.5 μg/0.1 ml of purified DNA to 0.9 ml of a fresh competent culture (Anagnostopoulos and Spizizen, 1961). The number of recipient cells was 2 x 10^8 bacteria/ml. The mixture was incubated for 30 min at 37°C with gentle shaking, DNase at 20 μg/ml was added and the cells were plated on the appropriate selective media.

Restriction enzyme analysis and DNA blotting

Purified DNA from Ncd, parental strains or φ105 phage were digested by the restriction enzyme *Eco*RI under conditions specified by the manufacturer. Electrophoresis of digested DNA was carried out on 1% agarose gels in 40 mM Tris-HCl buffer pH 7.8 with 2 mM EDTA, 20 mM sodium acetate. The DNA was blotted onto a Gene Screen Membrane provided by New England Nuclear Corp. (N.E.N.) and treated according to the conditions established by N.E.N.

Bacterial treatment before in situ hybridization

Well-isolated colonies derived from a cloned cell population were transferred with toothpicks to a selective medium to determine their phenotype and to a nitrocellulose filter. Both transferred colonies were grown overnight at 37°C. During growth, the bacteria transferred to the filter were in contact with a plate containing nutrient medium. After bacterial growth a lysozyme solution (20 mg/ml) was sprayed on the plates with a laboratory spray gun provided by Shandon, Southern Product Limited, Cheshire, UK. The lysozyme sprayed filters were incubated for 10 min at 37°C. Bacterial lysis and DNA treatment for colony hybridization were carried out according to Grunstein and Hogness (1975).

Hybridization procedure

φ105 DNA used as a probe was labelled by nick-translation using [α-^{32}P]CTP. The specific activity of the labelled probe was 1−3 x 10^8 c.p.m./μg (Rigby *et al.*, 1977).

Both kinds of filter containing either lysed colonies or DNA restriction fragments were hybridized with a labelled φ105 DNA probe according to the conditions described by Maniatis *et al.* (1982) for 18 h at 65°C.

The Gene Screen Membrane was washed following N.E.N. conditions. The nitrocellulose filters were washed as described by Maniatis *et al.* (1982).

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