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# Intraclonal polymorphism in the bacterium *Streptomyces ambofaciens* ATCC23877: evidence for a high degree of heterogeneity of the wild type clones

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## Abstract

In *Streptomyces ambofaciens* a genetic instability generates a high degree of polymorphism consisting of four main phenotypes: pigmented colonies (Pig<sup>+</sup> qualified as WT phenotype), pigment-defective colonies, pigmented colonies with pigment-defective sector and pigmented colonies with pigment-defective papillae. Molecular analysis of Pig<sub>col</sub><sup>-</sup> and Pig<sub>sec</sub><sup>-</sup> (pigment-defective mutant derived from a colony and a sector, respectively) produced by genetic instability and isolated in five Pig<sup>+</sup> subclones progenies revealed a new aspect of polymorphism in *S. ambofaciens* ATCC23877. Frequencies of deleted mutants deleted at the chromosome ends varied from one WT progeny to another. Two main types of deleted mutants were observed: deleted for one or both chromosomal extremities. The relative proportion of these two categories differed according to the WT progeny. These results argue for heterogeneity of the WT clones, i.e., Pig<sup>+</sup> colonies, originated from *S. ambofaciens* ATCC23877. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Polymorphism; Genetic instability; *Streptomyces*

## 1. Introduction

In *Streptomyces* bacteria, many characters are genetically unstable. This instability results from an important genome plasticity consisting of large deletions often associated with the amplification of adjacent DNA sequences. All genome rearrangements are located at the extremities of the linear chromosomal DNA [1–3].

In *Streptomyces ambofaciens* colony pigmentation is affected by the instability. This phenomenon leads to genetic polymorphism. Four phenotypes are mainly observed when *S. ambofaciens* is grown on complete medium: pigmented colonies (Pig<sup>+</sup>), pigment-defective colonies, pigmented colonies with pigment-defective sector, or pigment-defective papillae on the surface of the colony ([4,5], Fig. 1).

Molecular analysis of Pig<sub>col</sub><sup>-</sup> mutants showed large genome rearrangements, i.e. deletions of various size associated in some cases with amplifications of DNA sequences [6,7]. The rearrangements are located at the extremities of the chromosomal DNA and affect

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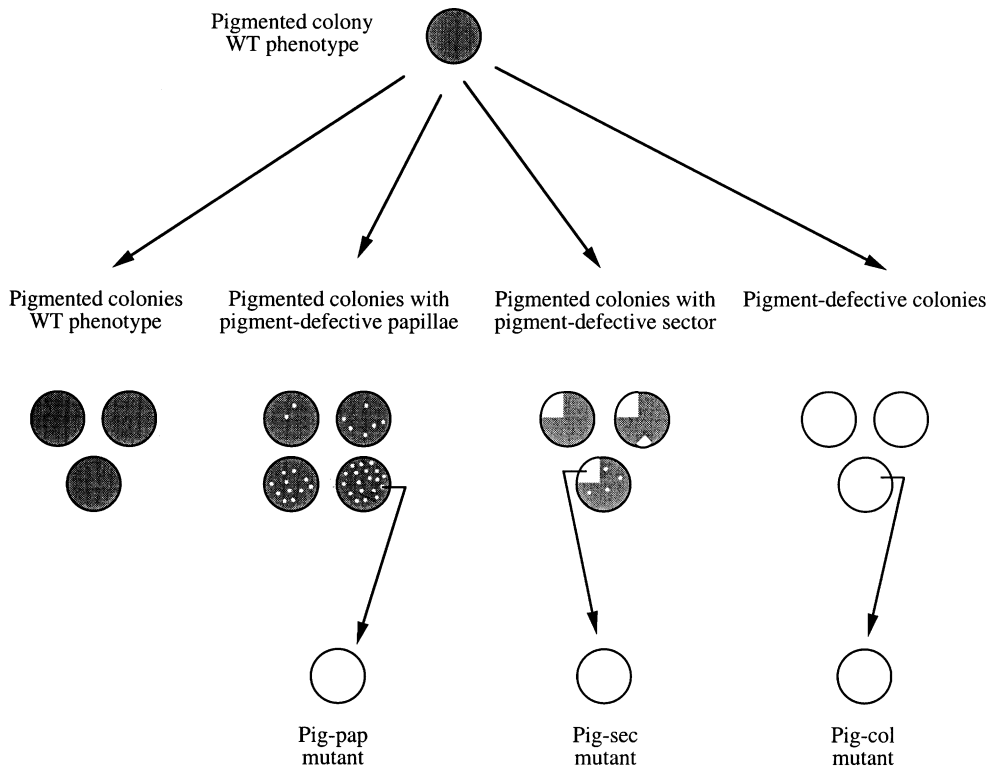


Fig. 1. Schematic representation of a WT progeny.

one or both chromosomal arms. Thus, different chromosomal structures are produced [3]. Thus, the  $\text{Pig}_{\text{col}}^-$  population, constituting a part of the genetic polymorphism observed in *S. ambofaciens*, is highly diversified. This constitutes a second aspect of polymorphism.

In a recent study of *S. ambofaciens* polymorphism, we demonstrated that colonies possessing different numbers of papillae were genetically different. In addition, colonies with a high number of papillae (called Hyperpap colonies), independently isolated in 12 WT progenies, corresponded to mutator strains. These mutators differed from each other and produced more  $\text{Pig}_{\text{col}}^-$  mutants and Hyperpap colonies than their ancestor [5].

In order to test whether the heterogeneity which existed among mutator strains was already present in the WT ancestors, we studied the genetic instability generated in the progeny of five WT subclones. In this paper, we show that frequency of deleted  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^-$  mutants (Fig. 1) depended on their WT

origin. In addition, the main type of deletion found in the  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^-$  populations (deletions for one or both chromosomal extremities) was determined by the WT origin of the mutants.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*S. ambofaciens* NSA2002 was derived from *S. ambofaciens* ATCC23877 [8] after 11 successive subclonings [9]. Strain NSA2002 was grown on Hickey–Tresner (HT) solid medium [10] at 30°C for 7 days with an average of 10 colony-forming-units (cfu) per plate. Five pigmented colonies without papillae or sectors were selected and subcultured once, via a confluent lawn, to provide five spore suspensions qualified as WT subclones: WT1, WT3, WT4, WT8 and WT12 [5]. The pigment-defective mutant strains studied in this work were derived from either pigment-defective colonies ( $\text{Pig}_{\text{col}}^-$ ) or

pigment-defective sectors ( $\text{Pig}_{\text{sec}}^-$ ) which arose after 14 days of growth at 30°C due to genetic instability in WT progenies. Mutants were isolated using a sterile toothpick and subcultured two, three or four times on HT solid medium until progenies were homogeneously pigment-defective.

## 2.2. Southern analyses

Total DNA was extracted from overnight cultures grown in HT liquid medium at 30°C as previously described [11]. Restriction enzymes were used under the conditions recommended by the supplier (Boehringer-Mannheim). DNA fragments were separated on agarose gels according to Sambrook et al. [12]. Southern blotting, prehybridization and hybridization conditions were used as previously described [13]. The cosmids used as probes were obtained from a genomic library of *S. ambofaciens* DSM40697 and correspond to specific regions of the *S. ambofaciens* genome as shown in Figs. 1 and 2A [11]. Cosmids 23E11 and 2B7 correspond to two loci containing Amplifiable Units of DNA, frequently amplified in *S. ambofaciens*, AUD90 and AUD6, respectively [7,13]. Labeling of probes and detection of specific hybrids were performed using the DIG DNA labeling and detection kit, under the conditions recommended by the supplier (Boehringer-Mannheim).

## 2.3. Pulsed-field gel electrophoresis (PFGE) analyses

DNA was prepared as previously described [11] from culture of *S. ambofaciens* strains grown at 30°C for 40 h in YEME (Yeast Extract Malt Extract) liquid medium [14]. PFGE runs were performed in a CHEF system (Biorad, USA, [15]). In all experi-

ments, 0.9% agarose gels were run in a 0.5 × TBE electrophoresis buffer. Pulse times varied according to the size of DNA fragments being separated.

## 2.4. Statistical analyses

A prerequisite for statistical comparison of frequencies of mutants is the independence of the mutational events leading to the production of mutants. A sector probably results from a mutation occurring during development of vegetative mycelium [4]. Consequently, each  $\text{Pig}_{\text{sec}}^-$  results from an independent mutational event. As described in the Results section,  $\text{Pig}_{\text{col}}^-$  were shown to be heterogeneous. Therefore, they probably derive from independent mutational events.

To compare two frequencies, we calculated the probability of obtaining the difference observed between the two samples if they had come from the same population, according to the comparison of two percentages in the case of small samples [16]. Probability values (*P* values) of  $\leq 0.05$  indicated that samples were significantly different from each other and that the difference was not likely due to sampling fluctuations. To compare more than two frequencies, a Chi-square test was used. It must be emphasized that a Chi-square test was only used when theoretical values were superior to 5.

## 3. Results

### 3.1. Localisation of inserts of recombinant cosmids used as probes on strain ATCC23877 chromosomal DNA

The probes used in this work derived from the strain DSM40697. G36, E7C, A85, 23E11 and 2B7

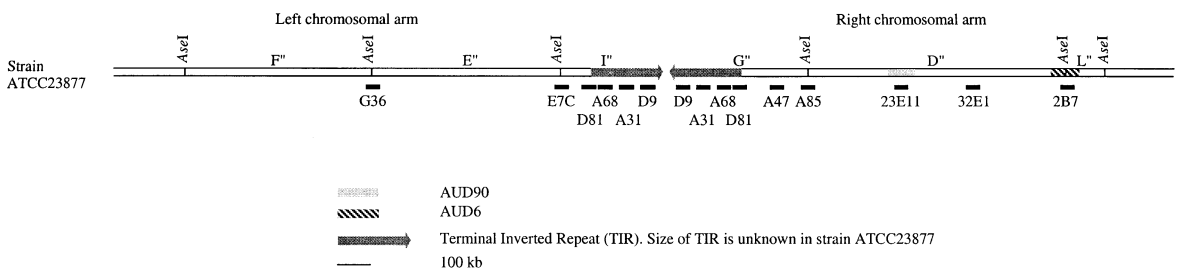


Fig. 2. *AseI* restriction map of the unstable region of the WT chromosomal DNA of strain ATCC23877 [11]. *AseI* fragment names and localization of probes used are indicated.

have already been localised to the DNA of strains DSM40697 and ATCC23877 ([11], Fig. 2). Regions homologous to probes A91, D9, 12A10, A31, A34, A68 and D81 were ordered in the terminal inverted repeat (TIR) of chromosomal DNA in strain DSM40697 [3]. All these probes cross-hybridized with strain ATCC23877 chromosomal DNA, except the terminal cosmid A91. The order of the regions revealed by D9, 12A10, A31, A34, A68 and D81 in strain ATCC23877 was shown to be the same as in strain DSM40697. Indeed, the 12A10 region overlapped the D9 and A31 regions similarly to the overlapping of the A34 region onto A31 and A68 regions. These probes also belonged to the TIR of strain ATCC23877 since all hybridized with both terminal *AseI* fragments. Finally, regions revealed

by the other probes used in this work were not ordered but their location on the right chromosomal extremity was verified. Thus, when hybridized with *AseI* restriction pattern, the probe A47 revealed the G'' fragment (Fig. 2) while probes 31G10, 8C4, 32E1, 6C7, 14C11 and 2B2 revealed the D'' fragment (Fig. 3A). This data is consistent with their position in strain DSM40697.

### 3.2. Strain ATCC23877 produced *Pig<sub>col</sub><sup>-</sup>* and *Pig<sub>sec</sub><sup>-</sup>* mutants non-deleted in the unstable region

Deletions were searched by hybridizing three recombinant cosmids (E7C, A85 and 23E11, Fig. 2) onto *Bam*HI restriction patterns of total DNA of *Pig<sub>col</sub><sup>-</sup>* and *Pig<sub>sec</sub><sup>-</sup>* mutants isolated in five WT proge-

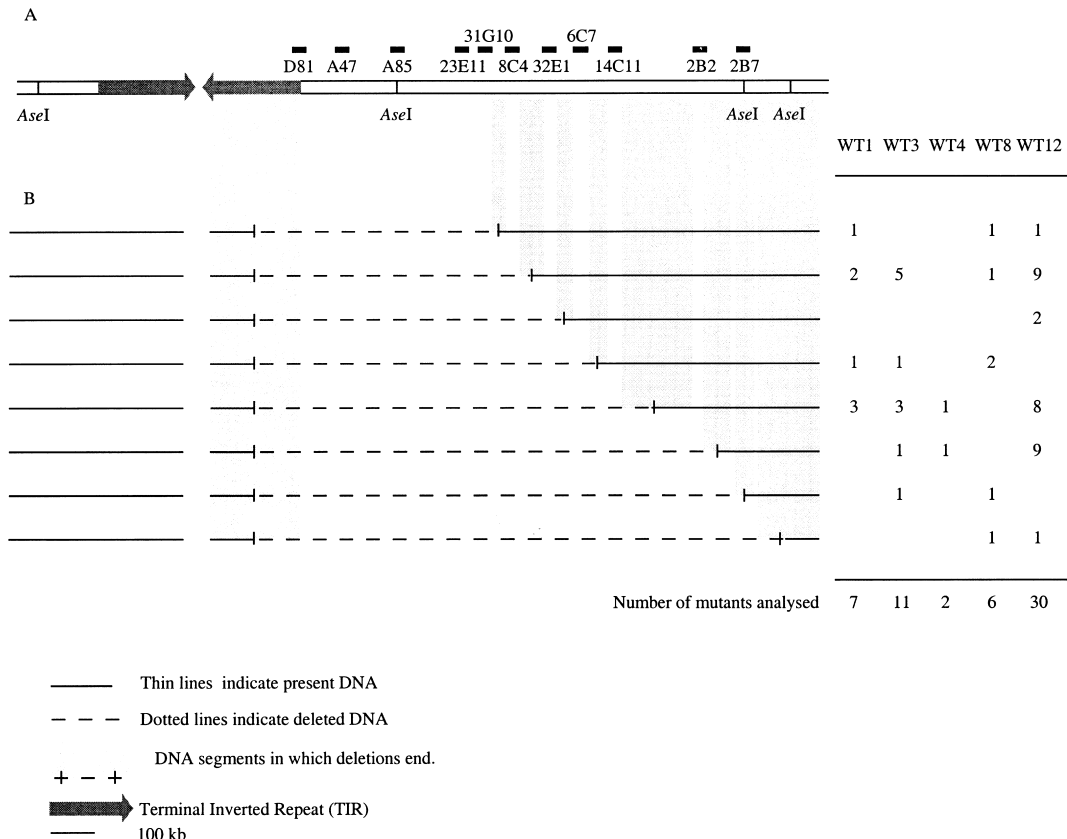


Fig. 3. *AseI* restriction map of the ends of the WT chromosome and representation of deletion in mutants. (A) *AseI* restriction map of the ends of the WT chromosome. Localization of recombinant cosmids used as probes. (B) Representation of DNA segments which comprise deletion points in 56 pigment-defective mutants deleted for the regions A85 and 23E11, deduced from hybridization results. Categories formed according to zones where deletions stopped, number of mutants belonging to each category and origin of mutants are also indicated.

nies (Fig. 1). These probes revealed regions belonging to the two chromosomal arms known to undergo rearrangements. In addition, their use allows detection of all the rearrangements observed in strain DSM40697 [11].

Numerous mutants without a deletion in the three regions tested were observed in each WT progeny (Table 1). In strain DSM40697, all the  $\text{Pig}_{\text{col}}^-$  mutants harboured a deletion [7]. Therefore, the possibility that the non-deleted pigment-defective mutants were composed of a mixture of deleted genomes and WT genomes was tested. The frequency of pigmented colonies ( $\text{Pig}^+$ ) was determined in the progeny of three undeleted  $\text{Pig}_{\text{col}}^-$  mutants: WT1 m10, WT3 m2 and WT4 m11. The frequency of  $\text{Pig}^+$  colonies observed in the progeny of WT1 m10 was 0.19% (1/841), 0.23% for WT3 m2 (4/1765) and 0% for WT4 m11 (0/447). The same segregation experiment was performed with three undeleted  $\text{Pig}_{\text{sec}}^-$  (1 derived from WT1 and 2 from WT3). The frequencies of  $\text{Pig}^+$  colonies observed in the progeny of these  $\text{Pig}_{\text{sec}}^-$  were 0% (0/2202), 0.05% (1/1891)

and 0.5% (7/1289), respectively. In order to test whether the weak proportions of  $\text{Pig}^+$  colonies observed could account for the numerous undeleted pigment-defective mutants observed, genomic DNA of WT4 and of a mutant deleted for the E7C region were mixed in a different ratio, digested with *Bam*HI and hybridized with E7C. A typical E7C hybridization pattern was observed for the patterns containing 50%, 20%, 10% and 5% of WT genome, but not for the profile containing 2% of WT genomes. Thus, a ratio of approximately one WT genome for twenty deleted genomes is necessary to obtain a positive signal with a probe. Therefore, the presence of a high frequency of non-deleted pigment-defective mutants could not be explained by a mixture of WT and deleted genomes.

As only three probes were used, mutants which appeared non-deleted could have deletions elsewhere. Hence, chromosomal DNA of two undeleted  $\text{Pig}_{\text{col}}^-$  (derived from WT4) and six undeleted  $\text{Pig}_{\text{sec}}^-$  (1 derived from WT1, 1 from WT3, 1 from WT4, 1 from WT8 and 2 from WT12) were analysed by

Table 1

Relative frequency of deleted  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^-$  mutants in the progeny of WT1, WT3, WT4, WT8 and WT12

The number of mutants deleted for the region revealed by the probes E7C, A85 and 23E11 is indicated. The number of amplified mutants is also shown. The relative frequency of deleted mutants corresponds to the number of deleted mutants among the mutants analysed in each WT progeny. These mutants were isolated in two independent experiments. The values result from these two experiments.

The number before <sup>a</sup>, <sup>b</sup> and <sup>c</sup> indicates the number of deleted mutants presenting the same type of amplification. *P*: probability that the relative frequency of deleted  $\text{Pig}_{\text{col}}^-$  mutants for each of the WT subclone was similar with that of deleted  $\text{Pig}_{\text{sec}}^-$  mutants.

WT origin	WT1		WT3		WT4		WT8		WT12	
	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$
Number of mutants non-deleted	11	60	14	17	32	26	16	52	11	53
Number of mutants deleted in the E7C region	7	2	14 <sup>1a</sup> <sub>1c</sub>	2	3 <sup>1a</sup>	0	9 <sup>2a</sup> <sub>2c</sub>	0	13 <sup>3a</sup> <sub>2c</sub>	1
Number of mutants deleted in the A85 region	0	0	2 <sup>1a</sup>	0	3 <sup>1a</sup>	0	0	0	0	1
Number of mutants deleted in the 23E11 region	0	0	1	0	1	0	0	0	1	0
Number of mutants deleted in the E7C and A85 regions	0	0	1 <sup>1c</sup>	0	1 <sup>1a</sup>	0	3 <sup>1a</sup>	0	1 <sup>1a</sup>	0
Number of mutants deleted in the E7C and 23E11 regions	0	0	1	0	0	0	1	0	1	0
Number of mutants deleted in the A85 and 23E11 regions	13	2	24 <sup>2b</sup> <sub>1c</sub>	8	6 <sup>1a</sup>	2	11	4 <sup>1a</sup>	36 <sup>1b</sup>	8
Number of mutants deleted in the E7C, A85 and 23E11 regions	0	0	2	0	2	0	0	0	1	1
Relative frequency of deleted mutants	20/31	4/64	45/59	10/27	16/48	2/28	24/40	4/56	53/64	11/64
Frequency of deletion $\times 10^2$	65	6	76	37	33	7	60	7	83	17
<i>P</i>	< 0.001		< 0.001		0.007		< 0.001		< 0.001	

<sup>a</sup>Amplification in the AUD90 region.

<sup>b</sup>Amplification in the AUD6 region.

<sup>c</sup>Amplification in an unknown region.

PFGE. All of these presented a WT *AseI* restriction pattern (data not shown). To test the possibility that these presented a small deletion not detectable by PFGE, *BamHI* restriction patterns of 28 undeleted  $\text{Pig}_{\text{col}}^-$  (6 arising from WT1, 9 from WT3, 10 from WT4 and 3 from WT8) were hybridized with eight additional probes belonging to the unstable region, 2B7, 32E1, A47, D81, A68, A31, D9 and G36 (Fig. 2). The same experiment was performed with 47 undeleted  $\text{Pig}_{\text{sec}}^-$  (9 derived from WT1, 11 from WT3, 10 from WT4, 8 from WT8 and 9 from WT12). One  $\text{Pig}_{\text{col}}^-$  which derived from WT3, was deleted for the A47 region, and was partially deleted in the 2B7 region. For the others, all the hybridization patterns obtained (74/75) were the same as the WT pattern. Thus, it could be concluded that almost all mutants which appeared to be non-deleted in the unstable region, were effectively non-deleted.

Thus, strain ATCC23877 produces, at high frequency,  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^-$  mutants that are non-deleted in the unstable region.

### 3.3. Heterogeneity of deleted $\text{Pig}_{\text{col}}^-$ and $\text{Pig}_{\text{sec}}^-$

In deleted  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^-$  mutant populations originated from a WT clone, heterogeneity was observed since mutants produced were deleted for one, two or three regions revealed by the probes (Table 1). Besides, in most of the WT progeny, some of the deleted  $\text{Pig}_{\text{col}}^-$  also presented an amplified DNA sequence (Table 1).

Three kinds of amplifications were found. First, amplifications in the AUD90 region (AUD for Amplified Unit of DNA) were revealed by the presence at high copy number of two *BamHI* typical fragments (4.6 and 5.6 kb) and by hybridization with the cosmid 23E11 corresponding to a part of the AUD90 (Fig. 2). Second, amplifications in the AUD6 region were revealed by hybridizing the cosmid 2B7 including the AUD6 region on *BamHI* restriction patterns (Fig. 2). Third, amplifications in regions different from AUD90 and AUD6 were observed on *BamHI* restriction patterns in which some fragments were more intense than others and so were present at high copy number. Furthermore, the size of the Amplified Sequence of DNA varied from one mutant to another

in a range of 5.3 to 29.15 kb (data not shown). Finally, only one  $\text{Pig}_{\text{sec}}^-$  derived from WT8, presented an amplified DNA sequence.

### 3.4. Numerous $\text{Pig}_{\text{col}}^-$ and $\text{Pig}_{\text{sec}}^-$ mutants were deleted in the right chromosomal arm alone

As D9 revealed the nearest regions from the ends of strain ATCC23877 chromosomal DNA, it was used to determine whether chromosomal extremities were affected in mutants deleted solely for the A85 and 23E11 regions. Since D9 revealed the TIR, it is present on both ends of the chromosomal DNA. Therefore, a negative signal indicates the absence of the two copies of D9, showing that the two TIR are at least partially deleted. A positive signal could correspond to the presence of at least one of the two copies of the D9 region, showing the presence of at least one intact TIR. Finally, a partial deletion could result from the total deletion of a copy and a partial deletion of the second copy of D9 since this implicates only one event of deletion.

The D9 probe was hybridized on *BamHI* restriction patterns of 46  $\text{Pig}_{\text{col}}^-$  deleted solely in the A85 and 23E11 regions (11 derived from WT1, 16 from WT3, 4 from WT4, 6 from WT8 and 9 from WT12). For one of the  $\text{Pig}_{\text{col}}^-$  analysed, the two copies of D9 were absent. One  $\text{Pig}_{\text{col}}^-$  was partially deleted for the D9 region. A WT hybridization pattern was observed for the remaining 44  $\text{Pig}_{\text{col}}^-$ , showing that at least one copy of the D9 region was present. Analysis of three of them (WT1 m2, WT3 m3 and WT4 m2) by PFGE was performed in order to check that the left chromosomal extremity remained intact. *AseI* restriction patterns showed that while the G' and D' fragments were absent, the I' fragment containing the left chromosomal end was present in each case (Fig. 2). One additional fragment (of about 40 kb) homologous to the probe 2B7 and probably corresponding to the junction fragment created after deletion, was observed in WT4 m2. For the two other  $\text{Pig}_{\text{col}}^-$ , the junction fragments were either too small and had run off their gels, or were too large and so were retained in their wells. Thus, these junction fragments could not be observed (data not shown). Therefore, in these  $\text{Pig}_{\text{col}}^-$ , the left chromosomal arm was unaffected by deletion.

Sixteen  $\text{Pig}_{\text{sec}}^-$  mutants deleted for A85 and 23E11 regions (2 from WT1, 5 from WT3, 1 from WT8 and 8 from WT12) were analysed in the same manner. All possessed at least one copy of the D9 region.

In conclusion, these results showed that most of mutants deleted for the A85 and 23E11 regions were affected solely on the right arm. Previously, this type of mutant was presumed to have linear chromosomal DNA.

### 3.5. Numerous $\text{Pig}_{\text{col}}^-$ and $\text{Pig}_{\text{sec}}^-$ mutants were deleted for both chromosomal arms

Hybridization of the D9 probe on *Bam*HI restriction patterns of 29  $\text{Pig}_{\text{col}}^-$  which seemed to be deleted only in the E7C region (seven derived from WT1, 11 from WT3, three from WT8 and eight from WT12) was performed. No hybridization signal was observed in 26  $\text{Pig}_{\text{col}}^-$  whereas in the last three ones, one band present in the WT hybridization pattern was missing in each of the mutant patterns. In these cases one TIR was totally deleted while the other was partially deleted.

Hybridization of the D9 probe on *Bam*HI restriction patterns of four  $\text{Pig}_{\text{sec}}^-$  deleted only in the E7C region (two derived from WT1, one from WT3 and one from WT12) revealed that these four were totally deleted for the D9 regions.

These results showed that mutants deleted for the E7C region, i.e., those on the left chromosomal extremity, were also affected on the right arm.

### 3.6. The different pigment-defective mutants probably derived from independent mutational events

A comparison of the frequencies of the different types of mutants in WT strains requires demonstration that each type of mutant results from independent mutational events. Each  $\text{Pig}_{\text{sec}}^-$  results from independent mutational event since each derives from one colony (Fig. 1). Thus, a study of heterogeneity was performed with  $\text{Pig}_{\text{col}}^-$  mutants only. A heterogeneity was firstly observed among the deleted  $\text{Pig}_{\text{col}}^-$  since they differed by the location of the deleted sequences and/or the size of amplified sequences (Table 1). However, in each WT progeny, the majority of the deleted mutants belonged to two cate-

gories: one comprises mutants deleted for A85 and 23E11 regions, and the other mutants deleted solely in the E7C region. Hence, it was necessary to determine whether a heterogeneity also existed among these mutants. Therefore, we searched for DNA segments containing the end of the deletion in these  $\text{Pig}_{\text{col}}^-$  (Table 1).

The experiment was performed with 56  $\text{Pig}_{\text{col}}^-$  deleted in the A85 and 23E11 regions (seven arising from WT1, 11 from WT3, two from WT4, six from WT8 and 30 from WT12). Nine probes revealing regions belonging to the right chromosomal arm (Fig. 3) were hybridized with *Bam*HI restriction patterns of the 56  $\text{Pig}_{\text{col}}^-$ . Eight types of deleted mutants were observed according to the sequences found to be deleted. Only zones where deletions stopped were determined. Thus, more categories could probably have been established if exact deletion points had been determined. Finally, heterogeneity was observed for the  $\text{Pig}_{\text{col}}^-$  isolated from a same WT progeny (Fig. 3).

The same experiment was realised with  $\text{Pig}_{\text{col}}^-$  deleted for the E7C region. *Bam*HI restriction patterns of 20  $\text{Pig}_{\text{col}}^-$  initially found to be deleted only for the E7C region (five derived from WT1, six from WT3, two from WT8 and seven from WT12) were hybridized with five probes in which four corresponded to regions of the TIR and one between the TIR and A85 region (Fig. 4), in order to roughly locate the deletion point on the right chromosomal arm in each  $\text{Pig}_{\text{col}}^-$ . Eight types of mutants were observed (Fig. 4). For the same WT progeny, different types of deleted  $\text{Pig}_{\text{col}}^-$  were observed. These results underlined the heterogeneity of  $\text{Pig}_{\text{col}}^-$  deleted in the E7C region. As for  $\text{Pig}_{\text{col}}^-$  deleted for A85 and 23E11 regions, heterogeneity is probably more important than initially perceived because only segments containing deletion points were determined.

In conclusion, most  $\text{Pig}_{\text{col}}^-$  mutants present different deletion sizes. To explain this heterogeneity, the simplest hypothesis considers that all these mutants result from independent mutational events. Finally, the deleted  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^-$  can be grouped into two categories: those deleted for the right chromosomal arm (deleted for A85 and/or 23E11) and those deleted for the two chromosomal arms (deleted for E7C, for E7C and A85 and for E7C, A85 and 23E11).



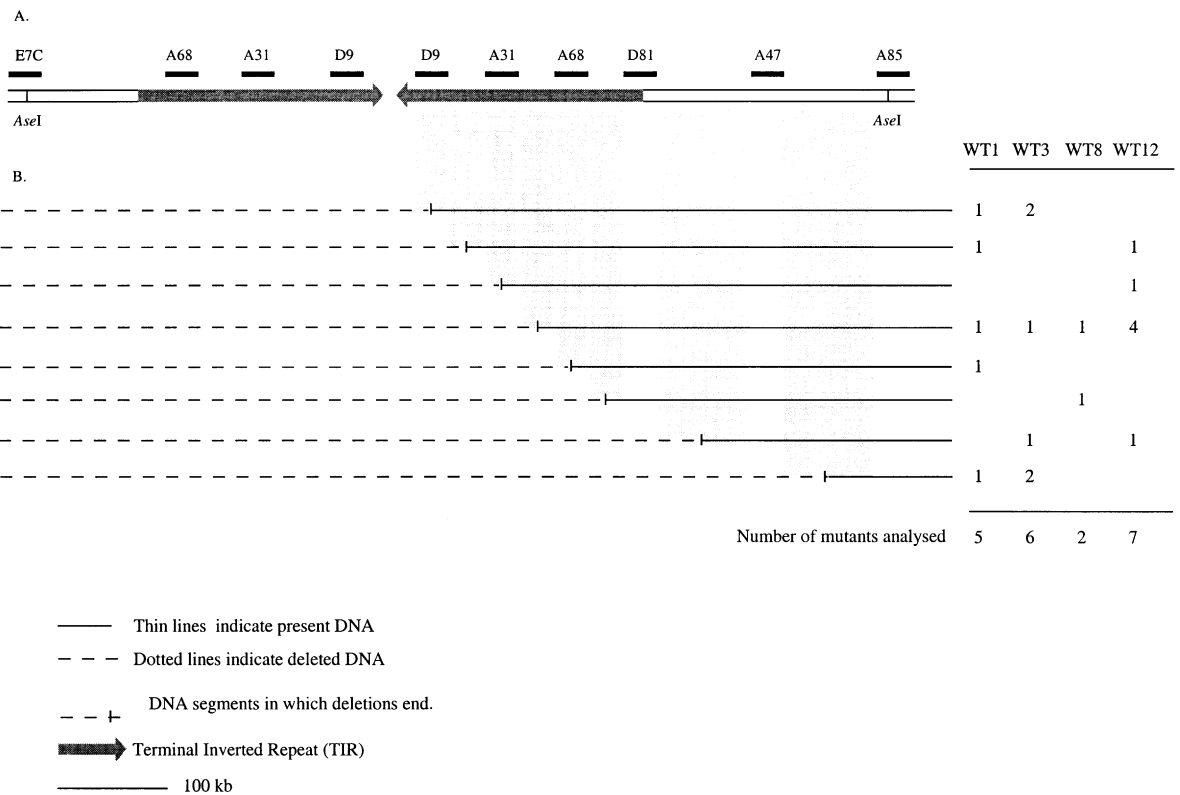


Fig. 4. *AseI* restriction map of the ends of the WT chromosome and representation of deletion in mutants. (A) *AseI* restriction map of the ends of the WT chromosome. Localization of recombinant cosmids used as probes. (B) Representation of DNA segments which comprise deletion points in 20 pigment-defective mutants deleted for the E7C region, deduced from hybridization results. Categories formed according to zone where deletions stopped, number of mutants belonging to each category and origin of mutants are also indicated.

### 3.7. Frequencies of deletion in $Pig_{col}^-$ and $Pig_{sec}^-$ mutants varied from one WT subclone to another

As shown in Table 1, the relative frequency of deletions in the  $Pig_{col}^-$  mutants varied from 33% to 83% depending upon their WT origin. The probability that the difference observed between the five WT subclones was due to a sampling fluctuation is less than 0.001 ( $\chi^2 = 33.94 > \chi_{4df}^2 = 18.467$ ). In the same manner, the frequency of deleted  $Pig_{sec}^-$  varied depending on the WT strain (Table 1). Thus, among  $Pig_{sec}^-$  arising from WT1, only 6% showed a deletion whereas among those deriving from WT3, 37% were deleted for at least one region. The difference observed between the five WT subclones was shown to be significant ( $\chi^2 = 19.89 > \chi_{4df}^2 = 18.467$ ,  $P < 0.001$ ). Moreover, in each WT progeny, the fre-

quency of deletion was shown to decrease significantly in  $Pig_{sec}^-$  with respect to  $Pig_{col}^-$  (Table 1).

### 3.8. The production of $Pig_{col}^-$ and $Pig_{sec}^-$ deleted for one or two chromosomal extremities varied from one WT subclone to another

As previously shown, the different deleted mutants could be grouped into two categories: those deleted for the two chromosomal extremities and those affected on the right chromosomal arm (Table 2). The proportion of each of these two categories among the  $Pig_{col}^-$  populations analysed was shown to differ according to their WT origin. The two types of mutants seemed to occur in the same ratio among the deleted  $Pig_{col}^-$  derived from WT4 and WT8 ( $P = 0.11$  and 0.22 respectively). Conversely, in the  $Pig_{col}^-$

Table 2

Number of mutants deleted in right or both chromosomal arms among  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^-$  obtained in the progeny of WT1, WT3, WT4, WT8 and WT12

$P$ : probability that the relative frequency of  $\text{Pig}_{\text{col}}^-$  or  $\text{Pig}_{\text{sec}}^-$  mutants deleted for the right chromosomal arm was similar with the frequency of  $\text{Pig}_{\text{col}}^-$  or  $\text{Pig}_{\text{sec}}^-$  mutants deleted for the two chromosomal extremities. For WT1, WT4 and WT8 the number of  $\text{Pig}_{\text{sec}}^-$  deleted was too small to calculate a significant probability.

WT strains	WT1		WT3		WT4		WT8		WT12	
	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$	$\text{Pig}_{\text{col}}^-$	$\text{Pig}_{\text{sec}}^-$
Number of analysed mutants	31	64	59	27	48	28	40	56	64	64
Number of deleted mutants	20	4	45 <sup>a</sup>	10	16	2	24 <sup>a</sup>	4	53 <sup>a</sup>	11
Number of mutants deleted in right chromosomal arm	13	2	27	8	10	2	11	4	37	9
Number of mutants deleted in both chromosomal arms	7	2	17	2	6	0	12	0	15	2
$P$	0.043	–	0.019	0.011	0.11	–	0.22	–	< 0.001	0.043

<sup>a</sup>The missing mutant is a mutant deleted for E7C and 23E11. Therefore it was not considered for the analysis.

arising from WT1, WT3 and WT12, mutants deleted on the right chromosomal arm occurred with the highest frequency ( $P = 0.043$ , 0.019 and 0.000015, respectively). Among the  $\text{Pig}_{\text{sec}}^-$  arising from WT3 and WT12,  $\text{Pig}_{\text{sec}}^-$  deleted for the right chromosomal arm occurred most frequently ( $P = 0.011$ , and 0.043, respectively). Thus, the same tendency was observed in  $\text{Pig}_{\text{col}}^-$  derived from WT3 and WT12.

In conclusion, the molecular analysis of  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^-$  mutants produced by genetic instability in five WT progenies suggests a heterogeneity of the WT clones.

## 4. Discussion

### 4.1. Polymorphism in *S. ambofaciens*

Although different isolates of the same species found in nature share numerous characteristics, a polymorphism is often observed. Thus, this work revealed a polymorphism in *S. ambofaciens* between strains ATCC23877 and DSM40697. Although both present common characteristics such as genetic and genomic instability, some differences were revealed in this study. Leblond et al. [7] showed that in  $\text{Pig}_{\text{col}}^-$  mutants produced by strain DSM40697, the A85 region was always deleted. Hence, the A85 region was qualified as a hot spot of deletion. Thus, all mutants deleted for the E7C region were also deleted for the A85 region. On the other hand, in strain

ATCC23877, mutants present a deletion for the E7C region without being affected in the A85 region. Nevertheless, in strain ATCC23877, the D9 region (on the right chromosomal arm) was shown to almost always be deleted in deleted  $\text{Pig}_{\text{col}}^-$ . Moreover, while 30% of  $\text{Pig}_{\text{col}}^-$  which derived from strain DSM40697 presented a deletion for the AUD6 region, only four  $\text{Pig}_{\text{col}}^-$  out of the 56 tested in this work (i.e., about 7%) were deleted in the AUD6 region. Thus, though both strains harbour a genomic instability, the hot spot of deletion, i.e., the region which is always deleted, differs in the two strains. In strain ATCC23877, the hot spot seems to be displaced towards chromosomal ends with respect to strain DSM40697. This difference of localisation could be explained by the fact that TIR sequences would be highly variable, and be responsible for a difference of chromosomal end structure between the two strains. This hypothesis is strengthened by the fact that the more extreme recombinant cosmid deriving from strain DSM40697 does not hybridize with strain ATCC23877 chromosomal DNA. Therefore, a region of about 30 kb is absent in strain ATCC23877 compared with strain DSM40697.

### 4.2. What is a wild type in *S. ambofaciens*?

A variation of the  $\text{Pig}_{\text{col}}^-$  (pigment-defective colony) and  $\text{Pig}_{\text{sec}}^+$  (pigmented colony with pigment-defective sector) frequencies was observed between the different WT subclones [5]. Molecular analysis of

$\text{Pig}_{\text{col}}^-$  mutants and  $\text{Pig}_{\text{sec}}^-$  mutants isolated in five WT subclone progenies revealed an important polymorphism between the WT subclones. A variation of the deletion frequencies at the chromosome ends was observed. Moreover, the relative proportion of mutants deleted for one or two chromosomal arms in each WT progeny could be pre-determined. These results suggest that genetic instability could be a dynamic phenomenon. Although the nature of this phenomenon is still unknown, recent data concerning mutator strains, which derived from these WT subclones, could facilitate understanding of the situation. These mutator strains present numerous pigment-defective papillae on their colonies and are induced in genetic instability, i.e., in the production of  $\text{Pig}_{\text{col}}^-$ . Frequencies of  $\text{Pig}_{\text{col}}^-$  and  $\text{Pig}_{\text{sec}}^+$ , as well as deletion frequencies in  $\text{Pig}_{\text{col}}^-$  also varied significantly between these strains. Since mutator strains are frequently observed in WT progenies (these represent about 10% of the WT progenies), WT subclones and mutator strains would not differ by classic mutations. Thus, the difference observed between the WT subclones and mutator strains could be due to different mutator states present in the spores from which they are derived. A low level of mutator state could be expressed in WT subclones while a high level would be present in mutator strains deriving from Hyperpap colonies. This suggests that spores from which WT colonies are derived could present different low mutator states. Up to now, external agents such as genotoxic agents (Ultra Violet light, mitomycin or oxolinic acid) were known to modify genetic instability levels [9]. This work suggests that internal agents, the nature of which is not yet known, could also influence the level of genetic instability. Besides, the wild type genome would present fast and dynamic evolution.

#### 4.3. Does a relationship exist between the stage of colony development and the occurrence of deletion events?

In the progeny of each WT strain, the frequency of deletion among the  $\text{Pig}_{\text{col}}^-$  was shown to be more important than that among the  $\text{Pig}_{\text{sec}}^-$ . We previously showed that  $\text{Pig}_{\text{pap}}^-$  (a pigment-defective mutant derived from papillae) did not present deletion [5]. The same observation was made with the amplifications.

Leblond et al. [4] suggested that these different types of mutants represented different aspects of the same mutational event occurring at different stages during colony development. This work specifies that the occurrence of deletion vary, depending on the origin of the mutant analysed. This raises the following question: Does the occurrence of rearrangements in *S. ambofaciens* depend on the morphological development of colonies?

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