Stimulation of genetic instability in *Streptomyces ambofaciens* ATCC 23877 by antibiotics that interact with DNA gyrase

JEAN-NICOLAS VOLFF, DOMINIQUE VANDEWIELE, JEAN-MARC SIMONET† and BERNARD DECARIS*

Laboratoire de Génétique et Microbiologie, Associé INRA, Faculté des Sciences de l’Université de Nancy I, BP 239, Institut de Biotechnologies, 54506 Vandoeuvre-lès-Nancy, France

(Received 16 February 1993; revised 18 May 1993; accepted 4 June 1993)

In wild-type *Streptomyces ambofaciens* ATCC 23877, pigment-defective (Pig−) mutants arise at a frequency of about 0.5%. This genetic instability is related to genomic rearrangements such as deletions and/or amplifications of DNA sequences. On media containing oxolinic acid and novobiocin, which interact with the A and B subunits of DNA gyrase, respectively, the frequency of variants increased dramatically. The Pig− mutant frequency was increased to almost 100% on a medium containing oxolinic acid at a concentration allowing 55% survival. On solid medium containing either oxolinic acid or novobiocin at subinhibitory concentrations, most colonies exhibited a 'patchwork' phenotype, characterized by the presence of numerous Pig− sectors. Similar phenomena were not observed on media containing the transcriptional inhibitor rifampicin or the translational inhibitor streptomycin. Many of the Pig− mutants exhibited a pleiotropic phenotype and were affected in aerial mycelium formation, colony growth and/or prototrophy. Moreover, the same kinds of rearrangements (deletions and/or amplifications of DNA sequences) were found in both induced and spontaneous Pig− mutants. The results suggest either that DNA gyrase is directly involved in genetic instability or that an SOS-like system is implicated.

Introduction

Genetic instability, i.e. the occurrence of spontaneous mutants at a frequency higher than 10⁻³, is frequently observed in the genus *Streptomyces* (for recent reviews, see Leblond et al., 1990; Birch et al., 1990). Traits affected by this phenomenon can differ from one species to another, and can include morphological and physiological differentiation, which are more complex in streptomycetes than in most other bacteria. Mutants arising from genetic instability often exhibit genomic rearrangements, such as deletion and/or amplification of DNA sequences. Some deletions found in streptomycetes are extremely large (Birch et al., 1989) and can affect about one quarter of the genome without affecting the survival of the bacteria (Leblond et al., 1991). In some cases, the chromosomal location of highly deletable genes has been reported (Leblond et al., 1990; Birch et al., 1990). Occasionally, a particular amplifiable unit of DNA (AUD) can be tandemly reiterated to yield several hundred copies of a particular amplified DNA sequence (ADS) per chromosome (Fishman & Hershberger, 1983; Hornemann et al., 1989). The presence of ADS has sometimes been related to particular phenotypes (Dittrich et al., 1991; Dary et al., 1992).

In two *Streptomyces ambofaciens* strains, DSM 40697 and ATCC 23877, pigment-negative (Pig−) mutants arise in the offspring of pigmented (Pig+) wild-type (wt) colonies at frequencies of 1 and 0.5%, respectively (Leblond et al., 1989; Volff et al., 1993). The Pig− mutant progeny are either phenotypically homogeneous or heterogeneous, i.e. representing a variety of phenotypes. The latter phenomenon has been called hypervariability (Leblond et al., 1989). In addition, Pig− mutants are affected more often than wt clones in such other traits as antibiotic production, aerial mycelium formation or UV resistance (Volff et al., 1993). The genetic instability of *S. ambofaciens* is related both to large deletions (Leblond et al., 1991) and to DNA reiterations, most of the latter being located in two regions, designated AUD90 and AUD6 (Demuyter et al., 1988). The AUD6 region is a hot spot for deletions (Demuyter et al., 1991). Moreover, an
ADS able to exist in both chromosomal and extrachromosomal ccc forms has been observed in a mutant isolated from strain DSM 40697 (Simonet et al., 1991). DNA sequencing of junctions generated after deletion (Birch et al., 1991) has not identified consensus sequences involved in genetic instability. Thus, the mechanism responsible for large genomic rearrangements at high frequency remains unknown.

In bacteria UV light, mitomycin C (MC) and nitrous acid induce deletions at frequencies that do not exceed $10^{-4}$ (Ishii & Kondo, 1975; Balbinder et al., 1983). These could be under the control of the SOS response (Ishii & Kondo, 1975; Balbinder et al., 1983). The SOS system, well described in Escherichia coli (for reviews, see Walker, 1985; Peterson et al., 1988), is likely to be present in streptomycetes (Stonesifer & Baltz, 1985; Misuraca et al., 1991). In S. ambofaciens ATCC 23877, the frequency of Pig' mutants is increased from $0.5\%$ to almost $30\%$ after UV light, MC or nitrous acid treatment, even at a high survival rate (Volf et al., 1993). Furthermore, the frequency of mutants harbouring ADSs and/or deletions at the AUD6 and/or AUD90 loci is increased from $0.4\%$ (spontaneous level) to more than $10\%$. These results suggest that the treatments induce genetic instability in S. ambofaciens by the induction of an SOS-like response.

The aim of this work was to determine whether the results obtained with the SOS inducers were due to interference with DNA replication, or whether any kind of stress could induce mutants at high frequency in S. ambofaciens. Thus, the influence on genetic instability of antibiotics inhibiting replication, transcription or translation was tested. Of the antibiotics used, oxolinic acid and novobiocin interact with the A and B subunits of DNA gyrase, respectively (Reece & Maxwell, 1991), rifampicin is an inhibitor of both RNA polymerase and transcription (Goldberg & Friedman, 1971) and streptomycin inhibits translation by binding to the 30S ribosomal protein S12 to prevent chain initiation and elongation (Pestka, 1977).

### Methods

**Bacterial strains, antibiotics, media and culture conditions.** Strain NSA2002 was isolated from Streptomyces ambofaciens ATCC 23877 (Pinnert-Sindico et al., 1954/55) after 11 successive subclonings of a wild-type Pig' Amy' colony. The other clones described in this paper were derived from strain NSA2002.

Oxolinic acid and rifampicin were from Sigma. Novobiocin was purchased from Boehringer Mannheim. Streptomycin was obtained as a gift from Rhône-Pouilenc Santé.

Colonies were grown at $30^\circ C$ on Hickey-Tresner (HT) complete solid medium (Pridham et al., 1956/57). For antibiotic treatments, spores of strain NSA2002 were plated on HT medium containing oxolinic acid (neutralized with NaOH), novobiocin, rifampicin or streptomycin. Experiments using novobiocin were carried out in very dim light. Colony diameters were determined after both 8 and 15 d of growth. Colony phenotypes were scored on the 15th day. After antibiotic treatments, colonies were subcloned three times on HT medium without antibiotic. The minimal medium (MM) used has been described by Smokvina et al. (1988). To determine auxotrophic requirements, MM was supplemented with either Casamino acids (Difco, 750 µg ml$^{-1}$) or with a single amino acid (37 µg ml$^{-1}$). Amino acid solutions were sterilized separately by autoclaving. For isolation of genomic DNA, strains were grown for 2 d in HT medium with agar omitted.

**Plasmids and DNA manipulations.** pOS15 was obtained by insertion of a 4.6 kb BamHI fragment into pIJ702 (Katz et al., 1983). This fragment originated from an ADS belonging to the AUD90 locus in a mutant of S. ambofaciens ATCC 23877.

pNSA6 was obtained by insertion of a 30 kb PstI fragment of DNA from S. ambofaciens DSM 40697 (Hütter, 1967) into the cosmid pIC79 (Hohn & Collins, 1980). This 30 kb fragment partially overlaps the AUD6 locus (Schneider et al., 1993). Total DNA isolation and restriction analyses were carried out as described by Demuyter et al. (1988). $^{32}$P labelling of DNA, Southern blotting and DNA--DNA hybridizations were done according to Demuyter et al. (1991).

### Results

**Oxolinic acid treatment**

To investigate the influence of oxolinic acid on genetic instability in S. ambofaciens, spores of strain NSA2002 were plated on HT medium containing oxolinic acid at concentrations from 0 to 10 µg ml$^{-1}$. For each concentration, the fraction surviving after 8 and 15 d of culture at 30°C was the same (Table 1). At 2.5 and 5 µg ml$^{-1}$, oxolinic acid had a very slight effect on survival; the surviving colony frequency was estimated to be $55 \times 10^{-2}$ at a concentration of 7.5 µg ml$^{-1}$ and was severely reduced at 10 µg ml$^{-1}$. (data not shown).

Colonies measured after both 8 and 15 d of culture were smaller on oxolinic acid-containing HT medium than on the control (Table 1). At a concentration

<table>
<thead>
<tr>
<th>Oxolinic acid concentration (µg ml$^{-1}$)</th>
<th>No. of colonies examined</th>
<th>Surviving fraction</th>
<th>Colony diameter (mm)</th>
<th>(10^2) Frequency of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>624</td>
<td>1.0</td>
<td>8.2 ± 0.1*</td>
<td>0.5</td>
</tr>
<tr>
<td>2.5</td>
<td>651</td>
<td>0.96</td>
<td>16.7 ± 0.2*</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>624</td>
<td>0.88</td>
<td>12.4 ± 1.1*</td>
<td>1.0</td>
</tr>
<tr>
<td>7.5</td>
<td>388</td>
<td>0.55</td>
<td>9.0 ± 0.2*</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The mean of diameter (±SE) of 10 colonies after 8 d.
† The mean of diameter (±SE) of 10 colonies after 15 d.
‡ Minimal and maximal colony diameters after 8 d.
§ Minimal and maximal colony diameters after 15 d.

Table 1. Effect of oxolinic acid on S. ambofaciens colony pigmentation.

In all cases the surviving fraction was the same after both 8 and 15 d of growth at 30°C. Phenotypes were scored after 15 d.
Table 2. Phenotypic characteristics of oxolinic acid- and novobiocin-induced Pig− mutants

Phenotypes were scored after three subclonings on antibiotic-free medium.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Inducing agent</th>
<th>No. observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amy+ Doc+ Aux+</td>
<td>Oxolinic acid</td>
<td>9+ +12+</td>
</tr>
<tr>
<td>Amy+ Doc+ Aux−</td>
<td>Novobiocin</td>
<td>9+ +10+</td>
</tr>
<tr>
<td>Amy− Doc+ Aux+</td>
<td>Oxolinic acid</td>
<td>1+ +12+</td>
</tr>
<tr>
<td>Amy− Doc+ Aux−</td>
<td>Novobiocin</td>
<td>1+ +12+</td>
</tr>
<tr>
<td>Amy− Doc− Aux+</td>
<td>Oxolinic acid</td>
<td>0+ +10+</td>
</tr>
<tr>
<td>Amy− Doc− Aux−</td>
<td>Novobiocin</td>
<td>0+ +0+</td>
</tr>
<tr>
<td>Amy− Doc− Aux+</td>
<td>Oxolinic acid</td>
<td>3+ +0+</td>
</tr>
</tbody>
</table>

* Pig− mutants from Pig− colonies on HT medium plus 7.5 μg oxolinic acid ml⁻¹, 25 mutants examined.
† Pig− mutants from Pig− sectors of patchwork colonies on HT medium plus 5 μg oxolinic acid ml⁻¹, 25 mutants examined.
‡ Pig− mutants from Pig− sectors of patchwork colonies on HT medium plus 0.10 μg novobiocin ml⁻¹, 11 mutants examined.
§ Pig− mutants from Pig− sectors of patchwork colonies on HT medium plus 0.15 μg novobiocin ml⁻¹, 14 mutants examined.

Some mutant clones observed in hypervariable offspring were phenotypically ‘stabilized’ by successive subcloning and gave homogeneous progeny. Eleven Pig− mutants isolated from the 11 homogeneous offspring and 14 stabilized Pig− mutants derived from the 14 hypervariable offspring were studied further (Table 2).

Among the 25 Pig− mutants examined, 15/25 totally lacked aerial mycelium on the surface of the colony (Amy−). Of these Pig− Amy− mutants, 11/15 showed a severely reduced colony diameter (Doc− phenotype). Of the 25 Pig− mutants, 21/25 were prototrophic. One of the four auxotrophic Pig− mutants unable to grow on MM was Pig− Amy− Doc+ and showed an arginine-supplementable leaky phenotype. The remaining three mutants displayed the more pleiotropic Pig− Amy− Doc− phenotype. One of these was Arg− (leaky phenotype on MM), the second grew well on MM plus Casamino acids (leaky phenotype on MM) and the third was unable to grow on MM supplemented with Casamino acids. Thus, 16 of the 25 mutants isolated exhibited a pleiotropic phenotype. This pleiotropy can be found in spontaneous Pig− mutants as well (Vollf et al., 1993).

On oxolinic acid-containing HT medium some colonies presented an heterogeneous phenotype, exhibiting numerous Pig− sectors that could be either Amy+ or Amy− (Fig. 1). The frequency at which this colony phenotype, called patchwork, was detected did not exceed 0.5 x 10⁻² at oxolinic acid concentrations of 2.5 and 7.5 μg ml⁻¹. At an intermediate oxolinic acid concentration of 5 μg ml⁻¹, patchwork colonies were observed at a frequency close to 100% (Table 1). Twenty-five Pig− sectors observed on 25 different patchwork colonies subcloned on oxolinic acid-free HT medium gave rise in all cases to Pig− progeny. In contrast, the offspring of 12 Pig+ Amy+ sectors from 12 different patchwork colonies were wt. These results indicated that Pig− sectors on patchwork colonies were probably due to mutations occurring during growth on oxolinic acid. Most of the sector-derived mutant offspring were phenotypically homogeneous (24/25), but one was hypervariable. Twenty-four Pig− mutants isolated from the 24 homogeneous progeny and one stabilized Pig− mutant obtained from the hypervariable offspring were analysed further: 12 of 25 mutants showed a Pig− Amy+ phenotype (Table 2), 13/25 were Pig− Amy−, and all of them were Doc+.

Most of the sector-derived mutants (24/25) were prototrophic, but one Pig− Amy− mutant (1/25 = 4 x 10⁻²) was unable to grow on MM plus Casamino acids (Table 2). Therefore, 13 out of the 25 Pig− mutants derived from Pig− sectors of patchwork colonies presented a pleiotropic phenotype.

To conclude, oxolinic acid induced or substantially simulated the occurrence of Pig− mutants. At a con-
centration of 7·5 µg ml\(^{-1}\), this phenomenon was especially pronounced. Oxolinic acid-induced Pig\(^-\) mutants could give rise to either homogeneous or hypervariable progeny and several pleiotropic phenotypes were observed among them. In these loss of mycelium pigmentation could be associated with lack of aerial mycelium and/or auxotrophy and/or reduced colony diameter. This is reminiscent of the pleiotropic effect of the spontaneous genetic instability in strain ATCC 23877 (Volff et al., 1993). Thus, oxolinic acid can induce or stimulate genetic instability in *S. ambofaciens* ATCC 23877.

**Novobiocin treatment**

When spores of the strain NSA2002 were plated on novobiocin-containing HT medium and incubated for 15 d at 30 °C, the fraction surviving was the same after either 8 d or 15 d of culture. A slight effect on survival was detected at 0·05, 0·075 and 0·1 µg ml\(^{-1}\) (Table 3); survival was reduced about threefold at a concentration of 0·15 µg ml\(^{-1}\). The fraction surviving on novobiocin at a concentration of 0·20 µg ml\(^{-1}\) was estimated to be 0·6 \(\times\) 10\(^{-2}\) (data not shown).

Novobiocin at 0·05 µg ml\(^{-1}\) had no significant effect on colony diameter, but at concentrations of 0·075 µg ml\(^{-1}\) and higher the colony diameter was reduced compared to the control (Table 3). The phenotype of colonies was scored after 15 d of culture at 30 °C. Novobiocin concentrations below 0·15 µg ml\(^{-1}\) did not influence significantly the frequency of Pig\(^-\) mutants, but at higher concentrations the frequency was increased about tenfold (Table 3).

At concentrations of 0·05 and 0·075 µg ml\(^{-1}\), patchwork colonies were detected at frequencies of 0·8 \(\times\) 10\(^{-2}\) and 1·2 \(\times\) 10\(^{-2}\), respectively (Table 3), whilst at concentrations of 0·10 and 0·15 µg ml\(^{-1}\), most of the colonies (91·8 \(\times\) 10\(^{-2}\) and 81·6 \(\times\) 10\(^{-2}\), respectively) presented a patchwork phenotype. This was reminiscent of the effect of HT medium plus 5 µg oxolinic acid ml\(^{-1}\).

To determine if these variant sectors were due to mutations affecting mycelium pigmentation, some of the Pig\(^-\) sectors observed on patchwork colonies were subcloned on antibiotic-free medium. Progeny of 11 Pig\(^-\) sectors from 11 different patchwork colonies grown on HT medium plus 0·10 µg novobiocin ml\(^{-1}\) and 14 Pig\(^-\) sectors from 14 different patchwork colonies grown on HT medium plus 0·15 µg novobiocin ml\(^{-1}\) were analysed further. The results are summarized in Table 2. In all cases (11/11 and 14/14) sector-derived progeny were Pig\(^-\). In contrast, six Pig\(^-\) Amy\(^+\) sectors from six different patchwork colonies observed on HT medium plus 0·10 µg

### Table 3. Effect of novobiocin on *S. ambofaciens* colony pigmentation

In all cases the surviving fraction was the same after both 8 and 15 d of growth at 30 °C. Phenotypes were scored after 15 d.

<table>
<thead>
<tr>
<th>Novobiocin concn (µg ml(^{-1}))</th>
<th>No. of colonies examined</th>
<th>Surviving fraction</th>
<th>Colony diameter (mm)</th>
<th>10(^2) \times) Frequency of colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>973</td>
<td>1·00</td>
<td>8·0±0·1*</td>
<td>Pig- Patchwork Pig+</td>
</tr>
<tr>
<td>0·05</td>
<td>779</td>
<td>0·88</td>
<td>15·2±0·1†</td>
<td>0·4</td>
</tr>
<tr>
<td>0·075</td>
<td>773</td>
<td>0·86</td>
<td>7·9±0·1*</td>
<td>0·3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15·3±0·1†</td>
<td>0·3</td>
</tr>
<tr>
<td>0·1</td>
<td>881</td>
<td>0·94</td>
<td>6·5±0·1*</td>
<td>0·1</td>
</tr>
<tr>
<td>0·15</td>
<td>251</td>
<td>0·30</td>
<td>14·1±0·2†</td>
<td>0·5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4·3±0·1*</td>
<td>0·1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12·1±0·2†</td>
<td>0·5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2·5±0·2</td>
<td>0·5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9·7±0·1†</td>
<td>0·5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5·6</td>
<td>12·8</td>
</tr>
</tbody>
</table>

* The mean of diameter (±SE) of 10 colonies after 8 d.
† The mean of diameter (±SE) of 10 colonies after 15 d.
novobiocin ml⁻¹ and six Pig⁺ Amy⁺ sectors from six different patchwork colonies found on HT medium plus 0·15 μg novobiocin ml⁻¹ all gave Pig⁺ Amy⁺ colonies. This showed that the numerous Pig⁻ sectors observed on patchwork colonies were the result of novobiocin-induced mutations.

Progeny of Pig⁻ sectors observed on HT medium plus 0·10 μg novobiocin ml⁻¹ could be either phenotypically homogeneous (7/11) or hypervariable (4/11). Eleven Pig⁻ mutants derived from these 11 offspring were isolated (Table 2). One of them presented a pleiotropic Pig Amy⁻ phenotype. In addition, one sector-derived Pig Amy⁺ mutant was unable to develop on MM, exhibited a leaky phenotype and grew on MM plus Casamino acids but not on MM plus arginine or methionine. The remaining 10 mutants were prototrophic. No Doc⁻ phenotype was found among the 11 mutants.

Pig⁻ sectors observed on medium plus 0·15 μg novobiocin ml⁻¹ gave rise either to homogeneous progeny (7/14) or to hypervariable offspring (7/14). Among the 14 Pig⁻ mutants isolated from these progeny, two were Amy⁻. Moreover, the 14 mutants presented a Doc⁺ phenotype.

Twelve of the 14 Pig⁻ mutants induced by novobiocin (0·15 μg ml⁻¹) were able to grow on MM but the remaining two could not. Both auxotrophs exhibited a Pig Amy⁻ phenotype. One of them exhibited a leaky Arg⁻ phenotype, whilst the other was unable to grow on MM plus Casamino acids.

To conclude, novobiocin substantially stimulated or induced mutations responsible for the Pig⁻ phenotype. Novobiocin-induced Pig⁻ sectors gave rise to either phenotypically homogeneous or hypervariable offspring. Furthermore, the pleiotropy of novobiocin-induced mutants affected both differentiation (mycelial pigmentation and/or aerial mycelium formation) and prototrophy. Like oxolinic acid, novobiocin can induce or stimulate genetic instability in S. ambofaciens ATCC 23877.

Rifampicin treatment

The survival rate of spores of strain NSA2002 plated on HT medium containing rifampicin and incubated for 15 d at 30 °C was the same after 8 and 15 d of culture at rifampicin concentrations from 0 to 50 μg ml⁻¹. The surviving fraction ranged from 0·95 x 10⁻² at 10 μg ml⁻¹ to 0·62 x 10⁻² at 50 μg ml⁻¹. At a concentration of 60 μg ml⁻¹, the fraction surviving was lower than 0·1 x 10⁻².

Colony diameters measured after both 8 and 15 d of growth were smaller for each rifampicin concentration than for the control.

Colony phenotypes were scored after 15 d of culture. On HT medium plus rifampicin, the frequency of Pig⁺ Amy⁺ colonies was over 99 x 10⁻². No significant change in Pig⁻ frequency relative to controls was found, and patchwork colonies were observed at frequencies ranging from 0·2 x 10⁻² to 0·4 x 10⁻². All Pig⁺ Amy⁺ colonies tested (50/50) gave rise to homogeneous Pig⁺ Amy⁺ colonies.

At all rifampicin concentrations tested, the colonies were smaller than those observed on HT medium plus 0·1 μg novobiocin ml⁻¹. Moreover, the colony diameter was smaller on HT medium plus 40 and 50 μg rifampicin ml⁻¹ than on HT medium plus 5 μg oxolinic acid ml⁻¹. Thus, even under more stringent growth conditions than on 0·1 μg novobiocin ml⁻¹ and 5 μg oxolinic acid ml⁻¹, rifampicin did not induce numerous Pig⁻ sectors in most of the colonies; however, patchwork colonies were detected at a frequency of about 0·3 x 10⁻². These results show that rifampicin does not significantly stimulate genetic instability.

Streptomycin treatment

For streptomycin concentrations from 0 to 1·25 μg ml⁻¹, the survival rate of spores of strain NSA2002 did not change between 8 and 15 d of culture. No significant effect on the survival rate was observed on medium containing relatively low concentrations of streptomycin (0·25 and 0·5 μg ml⁻¹), but the fraction surviving was reduced at higher antibiotic concentrations (from 19% at 0·75 μg ml⁻¹ to 2% at 1·25 μg ml⁻¹). Moreover, the colony diameters on media with streptomycin were reduced compared to the control.

After 15 d of growth on streptomycin, the Pig⁻ frequency was similar to the control, and most colonies exhibited a Pig⁺ Amy⁺ phenotype. No patchwork colony was found. The 50 Pig⁺ Amy⁺ colonies subcloned on streptomycin-free HT medium gave rise to Pig⁺ Amy⁺ offspring; none of these offspring was hypervariable.

The fractions surviving on streptomycin at concentrations of 0·75, 1 and 1·25 μg ml⁻¹ were much lower than on oxolinic acid or novobiocin at the concentrations that induced or stimulated genetic instability. Moreover, the colony diameter on streptomycin (1·25 μg ml⁻¹) was of the same order of magnitude as on oxolinic acid or novobiocin at concentrations that induced patchwork colonies. Therefore, streptomycin had no detectable effect on the genetic instability of S. ambofaciens ATCC 23877.

Genome rearrangements in induced Pig⁻ mutants

In S. ambofaciens ATCC 23877, approximately 60% of spontaneous Pig⁻ mutants underwent genomic rearrangements such as amplification or deletion of DNA
sequences (Vollf et al., 1993). To determine whether induced Pig− mutants likewise harbour rearranged DNA sequences, pOS15 (AUD90 locus) and pNSA6 (AUD6 locus) were hybridized with Southern blots of BamHI-digested total DNA of clones isolated in this work. pOS15 and pNSA6 contain a 4.6 kb BamHI fragment belonging to the AUD90 locus and a 30 kb PstI fragment that partially overlaps the AUD6 locus, respectively. The results are summarized in Table 4.

The hybridization pattern of the NSA2002 (wt) strain using pOS15 as a probe presented the expected 4.6 kb fragment corresponding to the insert. Moreover, several additional hybridizing restriction fragments were found, suggesting the presence of reiterations homologous to the probe in the genome of the wt strain. Using pNSA6 as a probe, 11 hybridizing restriction fragments were detected, corresponding to adjacent fragments of the AUD6 locus (Schneider et al., 1993). The 37 wt clones tested exhibited no rearrangements in either AUD90 or AUD6 loci.

In induced Pig− mutants, several kinds of rearrangements were found using these probes: (i) partial deletion; only some fragments were missing, (ii) total deletion; no signal was detected, (iii) ADS generation; detected as signals stronger than in the wt pattern. Twenty-four Pig− mutants isolated from patchwork colonies on HT medium containing 5 μg oxolinic acid ml−1 were analysed. Most of them (16/24) harboured deletions and no rearrangement was detected in the others. In addition, no rearrangement was detected in 12 Pig+ clones isolated from 12 Pig+ Amy+ sectors of patchwork colonies. Finally, all Pig− mutants isolated on HT medium plus 7.5 μg oxolinic acid ml−1 presented deletions in both AUD6 and AUD90 loci (22/22).

Among Pig− mutants induced by 0.1 μg novobiocin ml−1, 7/11 presented no visible rearrangement in either the AUD6 or the AUD90 loci, and 4/11 showed deletions. One of them harboured both ADS and a deletion, showing that both kinds of rearrangements can occur together. Six Pig+ clones derived from six Pig+ Amy+ sectors of patchwork colonies presented wt patterns. In addition, most Pig− mutants isolated after novobiocin treatment at 0.15 μg ml−1 showed deletions (12/13), and three of them contained an ADS. No rearrangement was found in six Pig+ clones obtained after subcloning six Pig+ Amy+ sectors of patchwork colonies.

The results show that most novobiocin- and oxolinic acid-induced Pig− mutants contain the same kinds of rearrangements (i.e. deletions and ADSs) as spontaneous Pig− mutants. Furthermore, all induced Pig− mutants with deletions at the AUD6 locus totally lacked the AUD90 region. In addition, all undeleted or partially deleted mutants at the AUD90 locus presented no rearrangement in the AUD6 region (Table 4). These observations could be explained by a polarity of deletions as observed in strain DSM 40697 (Leblond et al., 1991; Demuyter et al., 1991).

### Discussion

Most spores (99.5%) of the wt strain S. ambofaciens NSA2002 had a homogeneous Pig+ Amy+ phenotype. However, Pig− mutants arose at a frequency of about 0.5%, showing the genetic instability affecting this strain. Both oxolinic acid and novobiocin induced numerous Pig− mutant sectors in more than 80% of colonies, giving rise to the heterogeneous patchwork phenotype. This induction of genetic instability was observed even at subinhibitory concentrations. Moreover, on 7.5 μg oxolinic acid ml−1, the frequency of Pig− mutants increased almost to 100%, even at a high survival rate (55%). The observations made with oxolinic acid and novobiocin were similar to those reported earlier from our laboratory (Vollf et al., 1993) concerning the induction of genetic instability in S. ambofaciens by UV light, MC and nitrous acid. None of these remarkable effects was detected on media containing either the transcriptional inhibitor rifampicin or the translational inhibitor streptomycin, although the concentrations used affected colony growth and survival at least as much as the oxolinic acid and novobiocin treatments. Induced Pig− mutants had features similar to the Pig+ mutants that arose spontaneously at a frequency of 0.5% in wt S. ambofaciens. Both induced and spontaneous Pig− mutants were either phenotypically homogeneous or hyper-

### Table 4. Genome rearrangement in oxolinic acid- and novobiocin-induced Pig− mutants

<table>
<thead>
<tr>
<th>Inducing treatment</th>
<th>Phenotype of original colonies</th>
<th>Locus*</th>
<th>AUD90</th>
<th>AUD6</th>
<th>No. of mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxolinic acid (5 μg ml−1)</td>
<td>Patchwork</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δt</td>
<td>+</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δt</td>
<td>Δp</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δt</td>
<td>Δt</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxolinic acid (7.5 μg ml−1)</td>
<td>Pig−</td>
<td>Δt</td>
<td>Δp</td>
<td>15+2†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δt</td>
<td>Δt</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novobiocin (0.10 μg ml−1)</td>
<td>Patchwork</td>
<td>+</td>
<td>+</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δp</td>
<td>+</td>
<td>1+12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δt</td>
<td>Δp</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novobiocin (0.15 μg ml−1)</td>
<td>Patchwork</td>
<td>+</td>
<td>+</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δt</td>
<td>Δp</td>
<td>8+3†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δt</td>
<td>Δt</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* +, No deletions; Δp, partial deletions; Δt, total deletions.
† Mutants harbouring ADS homologous to pNSA6 (AUD6).
‡ Mutants harbouring ADS homologous to pOS15 (AUD90).
variable, they could be affected not only in mycelium pigmentation, but in traits such as aerial mycelium formation and/or prototrophy, and most of them exhibited deletions and/or amplifications of DNA sequences. In particular, all the mutants induced by 7.5 μg oxolinic acid ml⁻¹ contained rearrangements at both the AUD6 and AUD90 loci. Deletions and amplifications of DNA sequences could explain the pleiotropic effects of this instability by affecting several genes or a regulatory gene.

The effects of both novobiocin and oxolinic acid could be due to their interaction with DNA gyrase, a type II topoisomerase involved in DNA replication (for review, see Reece & Maxwell, 1991). DNA gyrase is composed of A and B subunits; oxolinic acid interacts with the A subunit (Reece & Maxwell, 1991), whilst the target for novobiocin in streptomycetes (Thiara & Cundliffe, 1988, 1989) and other bacteria (Reece & Maxwell, 1991) is the B subunit. Since the only other agents presently known to induce genetic instability in streptomycetes, UV light, MC, nitrous acid and ethidium bromide (Crameri et al., 1986; Dary et al., 1992), also have DNA as the main target, it is conceivable that the induction of genetic instability in streptomycetes is specific to treatments interfering with DNA replication.

In some bacteria, inhibition of DNA replication induces the SOS response, giving rise to overexpression of genes belonging to the SOS regulon (for reviews, see Walker, 1985; Peterson et al., 1988); this can lead to the induction of deletions in E. coli and Salmonella typhimurium (Ishii & Kondo, 1975). Quinolone antibiotics (such as oxolinic acid) and coumarin antibiotics (such as novobiocin), as well as UV light and MC, can induce the SOS response in some bacteria (Osborne et al., 1988; Piddock & Wise, 1987). The SOS system is likely to be present in streptomycetes (Stonesifer & Baltz, 1984; Misuraca et al., 1991) and could likewise be involved in inducing deletions and thus in genetic instability.

A second mechanism that can account for the results obtained with oxolinic acid and novobiocin is the formation of deletions mediated by recA-independent recombination as catalysed by DNA gyrase (Miura-Masuda & Ikeda, 1990). In E. coli, the frequency of deletions is increased about sixfold by oxolinic acid, and temperature-sensitive gyrA mutants exhibited fewer deletions (Miura-Masuda & Ikeda, 1990). Of five recombination sites, three coincided with DNA gyrase cleavage sites (Ikeda et al., 1984). Since most of the spontaneous and oxolinic acid- or novobiocin-induced Pig- mutants reported above had deletions at both the AUD90 and AUD6 loci, the gyrase-mediated recombination model (Ikeda et al., 1981; Miura-Masuda & Ikeda, 1990) could explain these aspects of genetic instability in S. ambofaciens. Furthermore, the complexity of rearrangements related to genetic instability in S. glaucescens seems to be reminiscent of those resulting from gyrase-mediated illegitimate recombination (Birch et al., 1991). However, the gyrase cleavage site sequence remains unknown in streptomycetes, and no consensus sequence involved in deletion formation has been found (Birch et al., 1991). A further difficulty concerning the second mechanism is that DNA damage induced by UV light, MC, nitrous acid and ethidium bromide is not readily explained unless it is assumed that these agents can modify the DNA gyrase activity to stimulate the occurrence of deletions. The fact that ethidium bromide can affect the action of DNA gyrase (Reece & Maxwell, 1991) may be compatible with this hypothesis.

We are grateful to Graeme Tait and Laurence Nicolas for their help in preparing the manuscript. This work was supported by grants from Ministère de la Recherche et de la Technologie and from the Université de Nancy I. We thank the Société de Secours des Amis des Sciences for its financial support to J.N.V.

References


