Analysis of genome instability in *Streptomyces ambofaciens*

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Genetic instability in *Streptomyces ambofaciens* DSM 40697 is correlated with genomic instability characterized by multiple rearrangements (deletions and/or amplifications) occurring in a large unstable region. We have focused on one of the two amplifiable DNA loci which were mapped in this region: the amplifiable unit of DNA locus 6 (*AUD*). The nucleotide sequence of one *AUD* fragment of 1.9 kb reveals the presence of two open reading frames (*ORF1* and *ORF2*) on the basis of the typical *Streptomyces* base composition at each of the three positions within codons. *ORF1* shows some similarity with a gene encoding a regulatory protein. The presence of potential genes in this unstable locus was unexpected because deletions occurred with high frequency within this region in the genetic instability-derived mutant strains. However, transcription analyses by SI nuclease protection experiments on the wild-type strain showed transcription of both *ORF1* and *ORF2*. Moreover, the amplified strain reveals increased transcription of *ORF1* but no transcription of *ORF2*. The amplification therefore results in a switch in transcription. The unstable region of *S. ambofaciens* DSM 40697 therefore is not a ‘silent’ region because at least some loci are transcribed.

**Introduction**

Genetic instability is a well-documented phenomenon in *Streptomyces* species, that affects characters such as sporulation, melanin formation, antibiotic production and resistance (Hütter & Eckhardt, 1988; Leblond *et al.*, 1990). As described below, very few markers have been localized to the genomic regions subject to genetic instability. The mutants arising from genetic instability have been shown to carry large chromosomal deletions (Birch *et al.*, 1989; Demuyter *et al.*, 1991; Leblond *et al.*, 1991). In addition to deletions, amplified DNA sequences are found with high frequencies in such mutant strains (Cullum *et al.*, 1986; Hütter & Eckhardt, 1988). The nomenclature proposed elsewhere (Fishman & Hershberger, 1983) is used: ADS for amplified DNA sequence, and *AUD* for amplifiable unit of DNA.

Streptomyces ambofaciens* DSM 40697 exhibits two levels of genetic instability: (i) a basic one; and (ii) hypervariability, another aspect of this phenomenon which is related to DNA amplification (Leblond *et al.*, 1989). Deletions have been characterized in all mutant strains and amplifications in about 17% of all mutant strains (Leblond *et al.*, 1991). All amplified strains of *S. ambofaciens* carry ADSs belonging to two amplifiable loci: *AUD* and *AUD90*. Pulsed-field gel electrophoresis experiments have shown that these two *AUD* families are located within the same chromosomal region leading to the notion of the ‘unstable region’ in the *S. ambofaciens* genome (Leblond *et al.*, 1991). Furthermore, the ADSs are associated with deletions which remove part of the proximal copy(ies) of these ADSs (Demuyter *et al.*, 1991). *AUD6* constitutes a rearrangement hotspot (Demuyter *et al.*, 1991). Indeed, 20% of the unamplified mutant strains present a deletion endpoint in *AUD6*. Furthermore, 13% of the amplified strains carry an ADS belonging to this locus which could therefore contain a deletion and/or amplification signal.

The *AUD*-encoded functions remain unknown in most *Streptomyces* species although it has been shown (Dittrich *et al.*, 1991) that a chloramphenicol resistance determinant localized in an amplifiable and deletable region of *S. lividans* 1326 is predicted to encode a putative transmembrane protein. Furthermore, the 'un-
stable region' of the S. glaucescens chromosome contains the melC and strS genes (Birch et al., 1989). Several examples of antibiotic resistance genes are known which are located within AUDs (Potekhin & Danilenko, 1985; Horneamann et al., 1987), as is a mercury resistance determinant (Altenbuchner & Brüderlein, 1986). In S. lividans, genetic instability seems to be a two-stage process in which the initial chloramphenicol sensitivity mutation switches on a dramatic arginine requirement instability (Altenbuchner & Cullum, 1984; Flett & Cullum, 1987). The arginine gene argG is affected by this genetic instability. This gene was mapped to the 3 o'clock region of the S. coelicolor A3(2) genome (Kieser et al., 1992) in which very few markers were localized by classical genetic analyses and which is thought to be a 'silent' region. Comparison between the physical map of S. lividans (Leblond et al., 1993) and the physical and genetic map of S. coelicolor has shown that the S. lividans unstable region corresponds to the silent region of S. coelicolor mapped at 3 o'clock.

In this paper, we report the study of AUD6, which is a rearrangement hotspot in S. ambofaciens DSM 40697. The nucleotide sequence analysis of one fragment of AUD6 reveals the presence of two open reading frames (ORFs). The two ORFs are transcribed in the wild-type (wt) strain and one of them shows increased transcription in the amplified strain.

**Methods**

**Bacterial strains and plasmids.** S. ambofaciens DSM 40697 (Hütter, 1967) was used as the wt strain. The amplified mutant strain NSA6 (Demuyter et al., 1991), which is amplified for AUD6, was a spontaneous derivative of the wt strain. In the progeny of NSA6 a deamplified mutant strain (called NSA1641) was isolated whose deletion consists of the 19 kb BamHI fragment of AUD6.

Escherichia coli HB101 (Boyer & Roulland-Dussoix, 1969) and Escherichia coli SURE (Stratagene) (uurC umuC recB hsdR mcrA mcrB mrr endA) were the hosts for cloning. Plasmid pBluescript KS (Short et al., 1988) and cosmid pHC79 (Hohn & Collins, 1980) were used.

**Media and culture conditions.** Streptomyces strains were grown at 37 °C on plates of Hickey–Tresner (HT) medium (Pridham et al., 1956/57). Large-scale DNA isolation was carried out after growth for 48 h at 37 °C with shaking (200 r.p.m.) in YEME liquid medium (Hopwood et al., 1985) supplemented with glycerol (0.25%). E. coli strains were grown at 37 °C in LB liquid medium (Maniatis et al., 1982). For growth curves and RNA isolations, JLP5 medium (J. L. Perrin, unpublished results) (yeast extract, 0.7%; NaCl, 0.5%; NaN3, 0.1%; MOPS, 2.1%, w/v; glycerol, 3.6%, v/v; pH 7.5) was inoculated with 5 x 10^6 c.f.u. after growth for 2 h at 37 °C in preincubation medium (casamino acid, 1%, w/v; yeast extract, 1%, w/v). The cultures were then incubated at 25 °C with shaking (200 r.p.m.). At each time point where RNA was prepared, DNA was also extracted and hybridization experiments revealed the same results as for the cultures at 37 °C. Furthermore, RNA isolations were performed after culturing at 25 °C because all studies in our laboratory on gene expression in the unstable region were done at this temperature (Dary and others, unpublished results).

**Growth curves.** Growth curves of the wt and mutant strains were obtained by following the DNA content of these strains. Samples (1 ml) were harvested from the cultures at different times. The mycelia were then sonicated using a Labsonic L apparatus (B. Braun), centrifuged and resuspended in 1 x SSC (0.15 M-sodium chloride, 0.015 M-sodium citrate). Colorimetric estimation of DNA was carried out using the diphenylamine reaction (Burton, 1956), with salmon sperm DNA as standard.

**Isolation of total DNA and restriction analyses.** DNA extraction and purification were performed as described by Demuyter et al. (1988). Restriction endonucleases were purchased from Boehringer Mannheim and used according to the manufacturer's recommendations. DNA fragments were electrophoresed on 0.8% agarose gels as described (Maniatis et al., 1982). Bacteriophage λ DNA digested with HindIII was used as a size standard (Daniels et al., 1983).

**RNA isolation and manipulation.** RNA was extracted from liquid cultures. RNA isolation and low-resolution S1 nuclelease protection experiments were performed as described by Hopwood et al. (1985). Forty micrograms of RNA were used in these experiments. The probe was the AUD6 19 kb BamHI fragment shown in Fig. 1. Bands on autoradiographs were quantified by densitometric analyses. RNA isolations were performed at different growth stages. The growth curves, RNA isolations and low-resolution S1 nuclelease experiments were done at least three times for each sample.

2P labelling of DNA. Southern blotting and hybridizations. DNA probes were 32P-labelled (Amersham) as described by Feinberg &
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Vogelstein (1983) using a nick-translation kit (Amersham) or a Megaprime kit (Amersham).

Southern blotting (Southern, 1975) was carried out by the capillary transfer method with the Vagucine system (LKB) onto Hybond-N membranes (Amersham). The DNA was depurinated with 0.25 M-HCl for 30 min before denaturation (in 1.5 M- Tris/HCl, 1.5 M-NaCl for 30 min) and neutralization (in 1.5 M-Tris/HCl, 1.5 M-NaCl for 30 min) and vacuum-transferred with 20 × SSC for 90 min. Prehybridization, hybridization and washing conditions have been described previously (Demuyter et al., 1988).

**Construction of a genomic library of S. ambofaciens DSM 40697.** Total DNA from S. ambofaciens DSM 40697 was partially digested with PstI to generate fragments of 35-40 kb. These were ligated into PstI-digested pHC79, which was then packaged by using the λ DNA in vitro packaging kit (Amersham). The cosmids were introduced into E. coli HB101 strain and recombinant clones were selected by in situ hybridization.

**DNA sequencing.** DNA sequences were determined by the dideoxy-chain termination method (Sanger et al., 1977) with pBluescript KS as vector. They were carried out with the TaqTrack sequencing system (Promega), using Taq DNA polymerase (Innis et al., 1988) for chain elongation. The nucleotide analogue 7-deaza-dGTP was substituted for dGTP in the reaction mixtures. Products of the reactions were analysed on 5% (w/v) polyacrylamide/7 M-urea gels. Nested deletions were carried out within the sequenced fragment using the double-stranded library of S. ambofaciens DSM 40697 was constructed in

**Results**

**Cloning of AUD6**

It has been shown (Demuyter et al., 1991) that AUD6 is a deletion hotspot and amplifiable locus in S. ambofaciens DSM 40697. To study AUD6 structure, a genomic library of S. ambofaciens DSM 40697 was constructed in pHC79 and in situ hybridization experiments using the cloned extremities of AUD6 (probe S1) (Demuyter et al., 1991) revealed one positive clone, containing a recombinant cosmids called pNSA6. This 40 kb-cosmid contained a part of AUD6 (Fig. 1). The AUD6 1.9 kb BamHI fragment was subcloned from pNSA6 (using E. coli SURE as host strain and pBluescript KS as cloning vector) to generate recombinant plasmid pNSA61. Hybridization experiments, using pNSA6 as a probe, revealed strong similarities with two other S. ambofaciens isolates: ATCC 23877 and ETH 11317 (data not shown), indicating the presence of this unstable region. The 1.9 kb fragment was present in these strains indicating that all the characteristics of this fragment are likely to be present in the ATCC and ETH isolates.

Hybridization experiments using pNSA61 as a probe revealed faint signals in the NSA6-amplified strain (data not shown) which were absent from strains DSM 40697 and NSA1641 (see Methods). These signals could thus correspond to a junction fragment produced as a result of the deletion. Thus, the right deletion endpoint in this strain could be localized within the 1.9 kb BamHI fragment. This fragment could therefore contain sequences involved in the amplification and/or deletion processes in S. ambofaciens.

**Nucleotide sequencing of the 1.9 kb BamHI fragment of AUD6**

The nucleotide sequence of the BamHI fragment is given in Fig. 2(a) and extends from nucleotides (nt) 1 to 1842 (G+C composition of 69% in good agreement with the high G+C content of the Streptomyces genome: 70–74%). Nucleotides 1843-2142 correspond to the beginning of the adjacent 3.5 kb BamHI fragment sequence (Fig. 1a).

The sequence contained two ORFs which showed base composition at each of the three positions within codons typical of that found for Streptomyces species (Bibb et al., 1984; Hopwood et al., 1986). Fig. 1(b) shows the localization of these two ORFs, which were on the opposite strands from each other, and the orientation of the 1.9 kb BamHI fragment in AUD6. ORF1 (nt 820–110) had a codon base composition of about 75, 43 and 90% G+C for the first, second and third positions of the codons, respectively, and began with ATG. This ORF potentially encodes a protein of 237 amino acids (Fig. 2a). In addition, there is a sequence (P1) resembling the Streptomyces consensus promoter sequence (Hopwood et al., 1986) immediately upstream of this ORF (Fig. 2b). No conventional ribosome-binding site was detected upstream of the ORF1 translational initiation codon. Although of rare occurrence, similar results were obtained for the aph gene of Streptomyces fradiae and for the ermE gene of Saccharopolyspora erythraea (Streptomyces erythraeus) (Bibb et al., 1985). Perhaps, this is a feature of the translational initiation of many Streptomyces genes.

ORF2 (nt 910–2118) overlapped the 1.9 kb BamHI fragment and the adjacent AUD6 3.5 kb BamHI fragment. This ORF was 1209 nucleotides long and presented a base composition of about 70, 46 and 95% G+C for the first, second and third positions within codons, respectively. This ORF was on the opposite strand from the first one. It began with GTG. Several nucleotides upstream of this GTG, a potential ribosome-binding site (AGGAG) was present. ORF2 potentially encodes a protein of 403 amino acids. A sequence (P2) resembling the consensus promoter sequence of Streptomyces was found upstream of this ORF (Fig. 2b).

The putative promoters P1 and P2 were located...
Fig. 2. Nucleotide sequence of the AUD6 1.9 kb BamHI fragment and 300 bp part of the adjacent 3.5 kb BamHI fragment. (a) Nucleotide sequence. Nucleotides 1–1842 corresponded to the 1–9 kb BamHI fragment sequence. Nucleotides 1843–2142 corresponded to the beginning of the adjacent 3.5 kb BamHI fragment sequence. The coding strand for each ORF, and both strands for the intergenic region and for the putative promoters are shown. Upper case letters correspond to the ORF1 and ORF2 sequences; lower case letters correspond to sequences outside of both ORFs. The amino acid sequence of ORF1 (nt 820–110) and ORF2 (nt 910–2118) is shown over their nucleotide sequence. The ORF2 putative ribosome-binding site is highlighted with asterisks. The ORF1 putative promoter P1 is boxed with thin lines (top strand), whereas the ORF2 putative promoter P2 is boxed with bold lines (bottom strand). The sequence resembling the gntR gene operator of the B. subtilis gluconate operon is overlined with a bracket. The arrows at the left represent the direction of transcription of both ORFs. Stop codons are indicated by St. (b) Comparison of the putative promoters located upstream of ORF1 (P1, middle line) and ORF2 (P2, bottom line) with the consensus sequence (top line) established for Streptomyces (Hopwood et al., 1986). Upper case letters represent the most conserved nucleotides in the consensus sequence. (c) Promoter region of ORF1 and ORF2. P1 is the ORF1 promoter and P2 is the ORF2 promoter.

between ORFs 1 and 2 (Fig. 2). Moreover, they were face-to-face. Transcription of ORF1 and ORF2 was divergent from these two putative face-to-face promoters. In the regions of divergent transcription with face-to-face promoters, the distances between the translational start sites range from 64 to 235 bp (Beck & Warren, 1988). The two translational start sites for AUD6 ORF1 and ORF2 were 89 bp apart and this region of DNA revealed an A + T content of 47%, which is high for Streptomyces.

**ORF analyses**

The amino acid sequences of both ORFs were compared with the NBRF protein sequence database using the FASTA program (Lipman & Pearson, 1985). The ORF1 predicted protein sequence revealed a 23.8% similarity (over 227 amino acids) with the Bacillus subtilis GntR protein (Fujita & Fujita, 1987), which is the repressor of the gluconate operon (Fig. 3a). Furthermore, analysis of the intergenic region revealed the presence of a sequence (located upstream of the translation initiation codon) which presented a strong similarity (14/16 nucleotides) with the operator region recognized by GntR in B. subtilis which is located upstream of the initiation codon of the gntR gene (Fujita & Miwa, 1989) (Fig. 3b). This region showed an imperfect dyad symmetry (Fig. 3b).

The ORF1 putative protein showed some features of DNA-binding proteins such as helix-turn-helix domains, one of which could be a good candidate for DNA binding, and which revealed a good alignment (with the FASTA program) with the predicted binding domains of GylR, the repressor of the S. coelicolor glycerol operon (Smith & Chater, 1988), AsnC (an activator of as-
paragine synthetase A transcription in *E. coli*: Dodd & Egan, 1987; Kölling & Lother, 1985), HutC (the repressor for the histidine utilization genes) of *Pseudomonas putida* (Allison & Phillips, 1990) and of *Klebsiella aerogenes* (Schwacha & Bender, 1990) (Fig. 4). The corresponding region of the *AUD6* ORF1 sequence also fulfilled the general criteria for forming the helix-turn-helix structure characteristic of many DNA-binding domains (Pabo & Sauer, 1984). These results are consistent with the proposal that the potential protein encoded by ORF1 could act as a regulatory protein. No homology with known sequences was found for the ORF2 predicted protein sequence.

**Transcriptional analysis**

Low-resolution S1 nuclease mapping was performed with RNA from the wt and the amplified NSA6 strains with the *AUD6* 1-9 kb *BamHI* fragment as the protected DNA. The negative control consisted of the NSA1641 strain in which the deletion included the 1-9 kb *BamHI* fragment.

With this 1-9 kb fragment probe, the transcript corresponding to ORF1 was expected to have a size of about 800–850 nt (taking into account the potential ORF1 promoter), and the ORF2 potential transcript was expected to have an overlap of about 1000 nt with the probe.

After hybridization of the unlabelled DNA probe with RNA isolated from different strains and S1 nuclease treatment, the potential DNA–RNA hybrids were subjected to alkaline electrophoresis and the remaining RNA extractions were prepared.

**Fig. 3.** Analysis of the predicted *S. ambofaciens* *AUD6* ORF1 protein. (a) Comparison of the predicted protein (*AUDSEQ*) with the *B. subtilis* gntR gene product (GntR). Colons represent identical amino acids, whereas points represent conservative changes as defined by Lipman & Pearson (1985). (b) Comparison of the operator sequence recognized by GntR (Fujita & Miwa, 1989) (top line) with the corresponding sequence of *AUD6* (bottom line and overlined in Fig. 2). The arrows indicate the dyad symmetry. The stars indicate the conserved nucleotides.

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**Fig. 4.** Comparison of the putative helix-turn-helix motif of the predicted *AUD6* protein with predicted helix-turn-helix motifs of different regulatory proteins. Colons and points are defined in the legend to Fig. 3. *AUD6* is the sequence of the putative helix-turn-helix domain of the *AUD6* ORF1. The position of this domain in the nucleotide sequence (Fig. 2) is indicated. GylR is the predicted sequence of the helix-turn-helix domain of the glycerol operon repressor of *S. coelicolor* (Smith & Chater, 1988). HutCPp and HutCKa are predicted sequences of the helix-turn-helix domains of the histidine operon repressors of *Pseudomonas putida* (Allison & Phillips, 1990) and of *Klebsiella aerogenes* (Schwacha & Bender, 1990), respectively. AnsC is the transcriptional activator of *asnA* in *E. coli* (Dodd & Egan, 1987; Kölling & Lother, 1985). The consensus residue types at each position of a typical helix-turn-helix DNA-binding domain (Pabo & Sauer, 1984) are indicated in the top line. G, predominantly glycine; H, usually non-polar; H', usually non-polar (alanine favoured); H", non-polar (usually valine or isoleucine); P, usually polar.

**Fig. 5.** Growth curves for the wt (□) and NSA6 (■) strains. The curves were obtained by following the DNA concentration during growth (see Methods). The black arrowheads indicate the time at which the RNA extractions were prepared.

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the wt strain and revealed a delayed growth compared to the wt strain. Furthermore, two other independently isolated AUD6 mutant strains showed the same delayed growth, whereas one AUD90 mutant strain without any rearrangement in AUD6 presented the same growth curve as the wt strain (data not shown). This delayed growth seemed to be a characteristic of mutant strains harbouring deletions in AUD6.

Fig. 6 shows the results of S1 nuclease protection experiments. With the ORF2-specific probe, two signals at about 1000 and 850 nt were visualized for both RNA preparations from the wt strain during stationary phase (Fig. 6, lane 1), but none for RNA extracted during exponential growth. Gel electrophoresis of RNA samples from exponential phase showed that the relevant RNA preparations were of suitable quality (due to the visualization of the rRNA bands and to the absence of smear). Thus, the absence of signals during exponential growth indicated an absence of transcription. Thus, ORF2 was transcribed during the stationary phase in the wt strain. In these experiments, the detected signals are the DNA corresponding to the overlap between RNA and DNA in the heteroduplex exposed to S1 nuclease. The 1000 nt transcript might correspond to that predicted from the ORF2 organization shown in Fig. 1(b) and from the DNA probe used in the S1 nuclease protection experiment (the 1-9 kb BamHI fragment). Two initiation sites for ORF2 transcription might exist, which could explain the two signals. Furthermore, another GTG translation start codon (in the same phase as the first one) was localized at nucleotide position 994 (Fig. 2). These two signals were undetected in the amplified strain (Fig. 6, lane 2) indicating that ORF2 transcription was abolished in this strain.

The ORF1-specific probe revealed a signal at about 850 nt in both RNA samples isolated during stationary phase from the wt strain (Fig. 6, lane 3). No signal was detected with the sample isolated during exponential growth. The transcript size of about 850 nt was in good agreement with that predicted from the nucleotide sequence. This transcript could not be the same as that of the ORF2 of the same size because there was insufficient homology (on the basis of the nucleotide sequence) between ORF1 and ORF2. Furthermore, the two ORF-specific probes did not give the same results in the amplified strain (see below). The same ORF1 probe used in the amplified strain revealed the same signal as that in the wt strain but it was much stronger (Fig. 6, lane 4). Thus, the amplified strain showed increased transcription of ORF1. Indeed, densitometric analyses revealed that the hybridization signal in the amplified strain was about 100-fold that in the wt strain. The amplified strain possessed an ADS copy number of 90. Thus, it seems that this increase in intensity corresponds to increased transcription associated with the amplification level. Increased transcription was observed for both RNA extractions prepared in stationary phase for strain NSA6. No transcription was observed for ORF1 in NSA6 during exponential growth. All the RNA samples (for both the wt and NSA6 strains) extracted during exponential growth showed a pattern similar to the one presented in Fig. 6, lane 5. Lane 5 shows the control with RNA not homologous to the probe (indeed, the genomic DNA of NSA1641 revealed no hybridization with the 1-9 kb BamHI fragment).

The 1-9 kb signal, still present in Fig. 6, lane 5, corresponded to the DNA–DNA probe renaturation. In conclusion, amplification of AUD6 might induce a switch in the transcription of both ORFs.

Discussion

The sequence of the 1-9 kb BamHI fragment, which contained a deletion end-point in strain NSA6, revealed the presence of two ORFs on the basis of the
Streptomyces
codon usage, which presents a bias due to
the high G+C content of the Streptomyces genome
(Wright & Bibb, 1992). The degree of synonymous
codon usage (SCU) bias of a gene is positively correlated
with its level of expression. The SCU of ORF1 and
ORF2 were similar to those of the 64 Streptomyces genes
studied by Wright & Bibb (1992), strongly indicating
that both ORFs might be expressed. Computer analyses
of the ORF1 product (homologies with the gluconate
operon repressor of B. subtilis, presence of a putative
helix-turn-helix motif) lead us to propose that ORF1
encodes a regulatory DNA-binding protein.

A sequence showing similarity to the consensus
promoter sequence of Streptomyces was present
upstream of the translation initiation codon of each ORF.
The ORF2 promoter did not contain the T residue at the
sixth position of the ‘−10’ box but three out of the six
nucleotides in this box are conserved (Fig. 2b). More-
over, not all the Streptomyces genes presented a
consensus promoter sequence.

The putative promoters P1 and P2 were face-to-face
and transcription of the two ORFs was divergent from
each of these two promoters. Such a situation has been
described for many bacterial and eukaryotic genes (Beck & Warren,
1988). A similar situation was found recently (Chen et
al., 1992) in S. lividans 66, where two ORFs of the
Tn4811 transposable element are orientated divergently.
Furthermore, the nucleotide sequence of a mercury
desistance determinant revealed six ORFs, organized in
two divergent units of four and two genes (Sedlmeier &
Altenbuchner, 1992). The two promoters are face-to-
face. Several other examples of promoters defining
complex regulatory regions are known in Streptomyces
(Janssen et al., 1989).

Low-resolution S1 nuclease protection experiments re-
vealed the transcription of the two ORFs in the wt strain
during the stationary phase. Furthermore, two trans-
cripts of different sizes could be synthesized from
ORF2. The amplified NSA6 strain showed no tran-
scription of the ORF2 but increased transcription of
ORF1. Further analyses would tell us if these genes were
translated and would give us information about the
according proteins. Insertional mutagenesis is now
being undertaken and could help us to determine the
function encoded by these ORFs. AUD6 was shown to
hybridize in the two other isolates of S. ambofaciens:
ATCC 23877 and ETH 11317. Thus, these two ORFs
might also exist in these isolates.

How are ORF1 and ORF2 regulated? In the wt strain,
the two ORFs were transcribed but amplification of this
region led to increased transcription of ORF1 (which
might be the regulatory gene) and to no transcription of
ORF2. The putative ORF1 gene product might therefore
inhibit transcription of ORF2 but not of ORF1. Such an
example exists in lysR-lysA genes of E. coli (Beck &
Warren, 1988 and references therein), where the LysR-
diaminopimelate complex activates lysA transcription
but LysR represses lysR transcription with or without
diaminopimelate. In this case, the same binding site
might be involved in both effects of the LysR product,
and it could imply another supplementary regulatory
molecule. In AUD6, the amplification led to the
symmetrical situation, where the transcription of the
regulatory gene was increased.

We have thus shown that the unstable region contained
genes. Recent studies (Leblond et al., 1993) have shown
that the unstable region of S. lividans corresponds to the
3 o'clock silent region of the S. coelicolor chromosome
(Kieser et al., 1992). Thus, the unstable region of
Streptomyces was expected to contain very few markers.
Our study demonstrated the presence of genes in the
unstable region of S. ambofaciens DSM 40697. Such
genes were not detected by classical genetic analyses and
perhaps might only be detectable by sequencing these
regions. Moreover, these genes could be deleted without
affecting significantly the viability of the strains under
laboratory conditions. Such a situation has been de-
scribed for chromosome III of the yeast Saccharomyces
cerevisiae on which 182 ORFs for proteins larger than
100 amino acids were detected (Oliver et al., 1992 and
references therein). Only a minor fraction of these ORFs
(34/182) has been identified by classical means and 80 %
show no significant homology to any previously se-
quenced genes. Thus, genome sequencing reveals new
functions that have not been discovered by classical
techniques.

Furthermore, the deletions and amplifications ob-
served in the mutant strains lead to new DNA sequence
associations. It would be interesting therefore to localize
the precise deletion end-point in the 1.9 kb BamHI
fragment in strain NSA6. Indeed, if this deletion end-
point was localized within ORF1, the new DNA
sequence, which originated from the left (in Fig. 1b) of
the AUD6 1.9 kb BamHI fragment, would become
dependent on the P1 promoter. Studies of such deleted
mutant strains might therefore allow the detection of
potential new genes.

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References

gene encoding the repressor for the histidine utilization genes of


Schwacha, A. & Bender, R. A. (1990). Nucleotide sequence of the...
gene encoding the repressor for the histidine utilization genes of *Klebsiella aerogenes*. *Journal of Bacteriology* 172, 5477-5481.


