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# Structural elements of the *Streptomyces oriC* region and their interactions with the DnaA protein

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**Streptomycetes differ from other prokaryotic organisms in their mycelial life cycle and in possessing a large, linear, GC-rich chromosome. To deduce structural features of the *Streptomyces* origin of chromosomal replication, the *oriC* sequences of three *Streptomyces* species (*S. antibioticus*, *S. chrysomallus* and *S. lividans*) were compared. In *Streptomyces*, the *oriC* region contains 19 DnaA boxes whose location, orientation and spacing are conserved. The consensus sequence of the DnaA box identified within *Streptomyces oriC* is (T/C)(T/C)(G/A/C)TCCACA (preferred bases underlined). The interactions of DnaA with DNA fragments containing single, two or three DnaA boxes were studied using surface plasmon resonance. The dissociation constant ($K_D$) for specific binding of individual DnaA boxes varied between 12 and 78 nM. *Streptomyces oriC* does not contain the three AT-rich 13-mer direct repeats present in the 5′ part of the *Escherichia coli oriC* region. However, short AT-rich sequences are distributed among the DnaA boxes of *Streptomyces oriC*. Repeated attempts to unwind *Streptomyces oriC* have been unsuccessful. It remains to be elucidated whether DnaA interacts with putative accessory proteins which help in unwinding *Streptomyces oriC*.**

Keywords: DnaA box, AT-rich sequence, *oriC*, *Streptomyces*

## INTRODUCTION

Streptomycetes are Gram-positive soil bacteria that grow as substrate mycelia differentiating to aerial mycelia and spores upon depletion of nutrients (Kützner, 1981). These bacteria differ from other prokaryotes not only in their mycelial life cycle but also in possessing a large (6–8 Mb), GC-rich chromosome, which has been found in linear form (Leblond *et al.*, 1993; Lin *et al.*, 1993; Lezhava *et al.*, 1995).

**Abbreviations:** BD, binding domain; GST, glutathione *S*-transferase.

The GenBank accession numbers for the sequences reported in this paper are AF026792 (*Streptomyces antibioticus*), AF027658 (*Streptomyces chrysomallus*) and M86491 (*Streptomyces lividans*).

In bacteria, chromosome replication is initiated at the replication origin, *oriC*, and the process is highly regulated (for review, see Kornberg & Baker, 1992; Messer & Weigel, 1996). The structure of the *oriC* region has been analysed within Gram-negative and Gram-positive bacteria. The sequences of *oriC* regions are conserved only among closely related organisms (Yoshikawa & Ogasawara, 1991). Sequence analyses have revealed that the origins of various eubacteria contain short, conserved sequences which are essential for *oriC* function: non-palindromic 9 bp sequences, so-called DnaA boxes and AT-rich regions (Yoshikawa & Ogasawara, 1991; Messer & Weigel, 1996). These conserved sequences are separated by spacer regions which vary in nucleotide composition and length. A

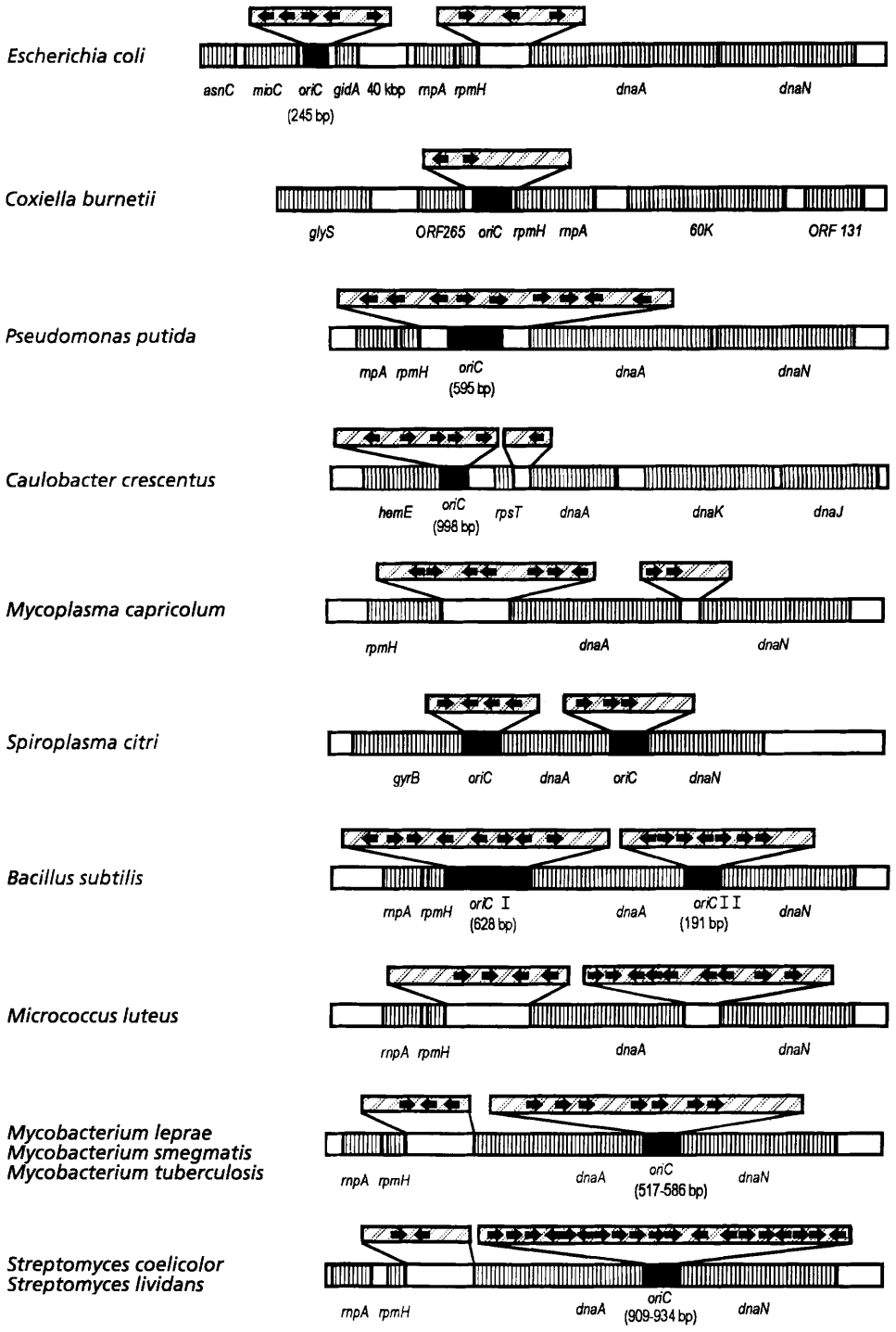


***Fig. 1.*** Comparison of the genetic organization of the *oriC* region of different bacteria. DnaA boxes are indicated by arrows. References: *Escherichia coli*, von Meyenburg & Hansen (1987); *Coxiella burnetii*, Shuan *et al.* (1994); *Pseudomonas putida*, Smith *et al.* (1991); *Caulobacter crescentus*, Marczyński & Shapiro (1992); *Mycoplasma capricolum*, Fujita *et al.* (1992); *Spiroplasma citri*, Ye *et al.* (1994); *Bacillus subtilis*, Yoshikawa & Wake (1993); *Micrococcus luteus*, Fujita *et al.* (1990); *Mycobacterium leprae*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, Salazar *et al.* (1996); *Streptomyces coelicolor*, Calcutt & Schmidt (1992); *Streptomyces lividans*, Zakrzewska-Czerwińska & Schrempf (1992), Zakrzewska-Czerwińska *et al.* (1994).

cluster of four or more DnaA boxes is an indication of a functional chromosomal origin. A putative *oriC* region from *Coxiella burnetii* contains only two DnaA boxes (Fig. 1) (Shuan *et al.*, 1994). However, it remains to be elucidated whether this region is able to promote autonomous replication. In *Bacillus subtilis*, two DnaA

box clusters are arranged upstream and downstream of the *dnaA* gene and act together as a replication origin (Fig. 1) (Yoshikawa & Wake, 1993). DnaA is the key protein in the initiation of DNA replication in bacteria (Kornberg & Baker, 1992; Skarstad & Boye, 1994; Messer & Weigel, 1996) and binds specifically to the DnaA box. The interaction of DnaA with its chromosomal origin is best understood in *Escherichia coli*. Five DnaA boxes are present within the *E. coli oriC* region. Binding of 10–20 DnaA monomers promotes a local unwinding of the AT-rich region (Bramhill & Kornberg, 1988; Messer & Weigel, 1996). The unwound region provides the entry site for the DnaB/DnaC helicase complex, followed by other proteins required to form a replication fork (Kornberg & Baker, 1992).

Most eubacteria contain a block of genes, *dnaA-dnaN-recF-gyrB*, encoding DnaA, the β-subunit of the DNA polymerase III holoenzyme, a product for recombination and the β-subunit of DNA gyrase, respectively (Messer & Weigel, 1996). Within many bacteria, including *B. subtilis* (Yoshikawa & Wake, 1993), *Micrococcus luteus* (Fujita *et al.*, 1990), *Mycobacterium* spp. (Salazar *et al.*, 1996), *Mycoplasma capricolum* (Fujita *et al.*, 1992) and *Pseudomonas putida* (Smith *et al.*, 1991), the *oriC* region is situated close to *dnaA* (Fig. 1). However, within some bacteria the arrangement is different. The *E. coli dnaA* region is located about 40 kb away from *oriC*. In *Coxiella burnetii*, *dnaA* is absent within the putative *oriC* region (Fig. 1) (Shuan *et al.*, 1994). The *Caulobacter crescentus oriC* is situated between *hemE* (encoding uroporphyrinogen decarboxylase) and *rpsT* (encoding a homologue of the ribosomal protein S20) and 2 kb away from *dnaA* which is separated (150 kb) from the *dnaN-recF-gyrB* region (Marczyński & Shapiro, 1992; Rizzio *et al.*, 1993; Zweiger & Shapiro, 1994). Sequences resembling other eubacterial *oriC* regions have not been detected in the vicinity of the *dnaA* genes of *Sinorhizobium meliloti* (Margolin *et al.*, 1995), *Synechocystis* sp. (Richter & Messer, 1995) and *Prochlorococcus marinus* (Richter *et al.*, 1998).

As in several other bacteria, the *oriC* region of *Streptomyces lividans* was identified as an autonomously replicating minichromosome (Zakrzewska-Czerwińska & Schrempf, 1992; Zakrzewska-Czerwińska *et al.*, 1995). It is situated between *dnaA* and *dnaN* and corresponds to the sequenced *oriC* region (Calcutt & Schmidt, 1992) of the closely related strain *S. coelicolor* A3(2). Recent discoveries suggest that the chromosome of *S. coelicolor* A3(2) replicates bi-directionally (Musialowski *et al.*, 1994) from the centrally located *oriC* (Radenbach *et al.*, 1996) and its linear form is assumed to be patched up at the ends by protein-primed replication (Chen, 1996).

In this paper we have compared the characteristics of *S. lividans oriC* with cloned *oriC* regions from *S. antibioticus* and *S. chrysomallus*, and we have determined interactions of individual DnaA boxes with *S. lividans* DnaA.

## METHODS

**Bacterial strains, culture conditions and transformation.** *Streptomyces* and *E. coli* strains used here are listed in Table 1. *E. coli* strains were grown in Luria–Bertani medium (Sambrook *et al.*, 1989). *Streptomyces* strains were cultivated on agar plates containing complete medium until sporulation occurred (Hopwood *et al.*, 1985). Spores were used to inoculate YEME liquid medium (Hopwood *et al.*, 1985). Propagation and transformation of *E. coli* and *Streptomyces* strains were carried out as described by Hopwood *et al.* (1985) and Sambrook *et al.* (1989). *Streptomyces* spp. were selected for resistance to 10 μg thiostrepton $ml^{-1}$.

**Plasmids and DNA library.** Plasmids are listed in Table 1. The *S. chrysomallus* genomic library in cosmid pV34 contains 32–34 kb DNA fragments obtained by partial *Sau*3A digestion of chromosomal DNA and cloned in the *Bam*HI site of the vector (Pahl *et al.*, 1992).

**Chemicals, enzymes and oligonucleotides.** Standard chemicals were obtained from Sigma or Serva. Restriction enzymes were supplied by Boehringer Mannheim, MBI Fermentas or Gibco-BRL. Oligonucleotides used for sequencing and PCR were chemically synthesized (MWG). For BIAcore studies, 5′ biotin-end-labelled oligonucleotides and their non-biotinylated complementary oligonucleotides were annealed by mixing equimolar amounts in 50 mM Tris/HCl, pH 7·5, 100 mM NaCl, 0·1 mM EDTA, heating to 85 °C and slowly cooling the samples to room temperature.

**DNA isolation and manipulation.** Total DNA was isolated from *Streptomyces* strains as described by Hopwood *et al.* (1985). Plasmid purification was done using a kit according to the manufacturer's protocols (Qiagen). The methods for the purification of DNA fragments, colony and Southern hybridization, and preparation of DNA probes have been described by Sambrook *et al.* (1989).

**DNA sequence determination and computer analysis.** DNA sequencing was performed using the dideoxy chain-termination method (Sanger *et al.*, 1977) with Sequenase (USB) and [α-$^{35}$S]ATP (Amersham). The nucleotide sequence was determined on both strands. Computer analysis was done using the GCG package programs for ORF identification and sequence alignment.

**PCR.** Comparison of the amino acid sequences of DnaA and DnaN allowed selection of highly conserved motifs at the C terminus of DnaA (FGGRDH) and the N terminus of DnaN (MKIRVER). Taking into account the known *Streptomyces* codon usage, two degenerate primers were deduced. The nucleotide sequences of the primers are as follows: $p_{dnaA}$, 5′ CGCGGATCCTTCGGSGGSCGSGACCAC 3′; $p_{dnaN}$, 5′ AACTGCAGSCGCTCSACSCGGATCTTCAT 3′ (S = G or C). Each of them was tailed by a motif for a restriction site. PCR was done with 2·5 U Dynazyme II DNA polymerase (Finnzymes) in 50 μl of the recommended buffer and was performed for 40 cycles (10 cycles of 1 min at 95 °C, 1 min at 50 °C and 1·5 min at 72 °C, and 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 1·5 min at 72 °C). Approximately 300 ng genomic DNA from *S. antibioticus* ETH 7451 was used for PCR. The amplified products were analysed on a 1% agarose gel, purified with the QIAquick PCR purification kit (Qiagen), digested with restriction enzymes (*Bam*HI, *Pst*I) and then cloned in pUO9090.

**Isolation of *oriC* fragments using affinity chromatography.** The DNA-binding domain (BD) of *S. lividans* DnaA was fused

***Table 1.*** Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype and/or relevant characteristic* | Reference |
|---|---|---|
| **Strains** | | |
| *E. coli* AG115 | *lacX74 galU galK araD139 strA hsdR17* F′ *lacI*$^q$ *lacZ*::Tn*5* | Mattern (1992) |
| *E. coli* DH5α | *supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | Sambrook *et al.* (1989) |
| *E. coli* TG1 | *supE hsdΔ5 thi* Δ(*lac–proAB*) F′ [*traD36 proAB*$^+$ *lacI*$^q$ *lacZ*ΔM15] | Sambrook *et al.* (1989) |
| *E. coli* WM2121 | *ara* Δ(*lac–pro*) *fis*::Km *recA56 rpsL srlC300*::Tn*10 thi* | Koch *et al.* (1988) |
| *S. antibioticus* | ETH 7451 | Novella *et al.* (1992) |
| *S. chrysomallus* | ATCC 11523 | |
| *S. lividans* TK21 | SLP2$^-$ SLP3$^-$ derivative of *S. lividans* 66 | Hopwood *et al.* (1985) |
| **Plasmids** | | |
| pBR322 | Ap$^r$, Tc$^r$ | Bolivar *et al.* (1977) |
| pUC18 | Ap$^r$ | Yanisch-Perron *et al.* (1985) |
| pUK21 | Km$^r$ | Vieira & Messing (1991) |
| pUO9090 | pUK21 derivative containing 1·5 kb Am$^r$ | M. C. Martin (unpublished) |
| pBluescript II SK(+) | pUC derivative (pMB1 replicon) | Short *et al.* (1988) |
| pLEX3BT | *tac* promoter, Ap$^r$ | Diederich *et al.* (1994) |
| pGEX-3X-6His | GST gene fusion vector, Ap$^r$ | Majka *et al.* (1997a) |
| pUSA1 | pUO9090 derivative containing 934 bp *Bam*HI–*Pst*I fragment of the *oriC* region of *S. antibioticus* | This study |
| pBSC1 | pBR322 derivative containing 4·2 kb *Bam*HI fragment of the *S. chrysomallus dnaA* region | This study |
| pBSC2 | pBluescript II SK(+) derivative containing 1228 bp *Sac*I–*Bam*HI fragment of the *oriC* region of *S. chrysomallus* | This study |
| pBSL1 | pBluescript II SK(+) derivative containing 1092 bp *Sph*I–*Bgl*II fragment of the *oriC* region of *S. lividans* | Zakrzewska-Czerwińska *et al.* (1995) |
| pGDnaA(BD) | pGEX-3X-6His derivative containing 432 bp *Xho*II–*Xho*II–*Sph*I fragment encoding the DNA BD of *S. lividans* DnaA | Majka *et al.* (1997a) |
| pLEXDnaA6xHis | pLEXBT derivative containing the entire *S. lividans dnaA* gene and $(CACCAT)_3$ encoding six histydyl residues at the 3′ end of *dnaA* gene | Majka *et al.* (1997b) |

* ATCC, American Type Culture Collection, Rockville, MD, USA. Am, apramycin; Ap, ampicillin; Km, kanamycin; Tc, tetracyclin.

to the C terminus of glutathione *S*-transferase (GST) as described previously (Majka *et al.*, 1997a). Plasmid pGEX-DnaA(BD), encoding the fusion protein GST-DnaA(BD), was transformed into *E. coli* AG115. Cells were grown for 3 h at 37 °C to an $OD_{550}$ of 0·6 in the presence of 100 μg ampicillin $ml^{-1}$ and then induced with 0·5 mM IPTG for 1·5 h. After centrifugation (5000 ***g***, 4 °C, 10 min), cells were resuspended in lysis buffer (50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, pH 8·0) and degraded by sonification (five times, 30 s each). After centrifugation (30000 ***g***, 4 °C, 60 min), the crude extract was treated with 25 μg DNaseI $ml^{-1}$ (37 °C, 30 min). The GST-DnaA(BD) fusion bound directly from the bacterial extract to the glutathione-Sepharose beads. The column was then washed with 10 bed volumes of lysis buffer. The DNA, digested with restriction endonuclease, was loaded onto the glutathione-Sepharose-GST-DnaA(BD) in 'low' salt buffer (20 mM Tris/HCl, 100 mM NaCl, pH 8·0). After 1 h incubation at room temperature, the column was washed with three column volumes of 'medium' salt buffer (20 mM Tris/HCl, 500 mM NaCl, pH 8·0) to remove DNA that did not tightly associate with the GST-DnaA(BD) beads. DNA that remained bound to the beads was then eluted with 'high' salt buffer (20 mM Tris/HCl, 2000 mM NaCl, pH 8·0), followed by 2-propanol precipitation. The DNA was re-suspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) and analysed on an agarose gel.

**Purification of *S. lividans* DnaA.** *S. lividans* DnaA was overexpressed in *E. coli* WM2121 as a His-tagged protein and then purified on a $Ni^{2+}$-NTA-agarose column (Qiagen) as described previously (Majka *et al.*, 1997b).

**Surface plasmon resonance.** The biotinylated double-stranded oligonucleotides were immobilized on a streptavidin-coated SM 5A chip of the BIAcore apparatus. Usually, 100–250 RU (resonance units) of DNA was immobilized. DNA loosely attached to the surface of the chip was removed with 0·05 % SDS. To exclude the effects of mass transfer on the kinetics of protein–DNA interactions, the measurements were performed at various protein concentrations (17·4–174 nM) and at three different flow rates (2, 5 and 10 μl $min^{-1}$). The calculated kinetic constants did not differ significantly. Thus, the subsequent measurements were performed at a continuous flow of 2 μl HBS buffer $min^{-1}$ (HBS buffer: 10 mM HEPES, pH 7·4, 150 mM NaCl, 3·4 mM EDTA, 0·005 % BIAcore surfactant P20). At the end of each cycle, bound DnaA was removed by washing with 0·05 % SDS. The BIAevaluation version 2.1 program (Pharmacia Biosensor) was utilized for data analysis.

## RESULTS AND DISCUSSION

### Cloning of the *oriC* regions of *S. antibioticus* and *S. chrysomallus*

Comparisons of the deduced amino acid sequences of DnaA and DnaN from several bacteria, including *S. lividans*, showed two highly conserved motifs in the respective C- and N-terminal regions. Two corresponding degenerate primers were deduced and used to amplify a DNA fragment (~ 900 bp) from total *S. antibioticus* DNA. The fragment was cloned into pUO9090 to yield plasmid pUSA1 (Table 1). Hybridization with totally digested chromosomal DNA proved that the cloned fragment was derived from *S. antibioticus* (data not shown).

To isolate the *oriC* region of *S. chrysomallus*, a library of its genomic DNA in the cosmid vector pV34 (Pahl *et al.*, 1992) was hybridized with a 1·2 kb *Bam*HI–*Sph*I fragment encoding the C terminus of *S. lividans* DnaA (Zakrzewska-Czerwińska *et al.*, 1994). From 12000 colonies, eight positive clones were obtained. Within each of the cosmids a 4·2 kb *Bam*HI fragment hybridized with the *dnaA* probe and cloning of this *Bam*HI fragment in pBR322 gave pBSC1. Sequence analysis allowed the identification of the 3′ end of *dnaA* and the 5′ end of *dnaN*. A 1·2 kb *Bam*HI–*Sac*I fragment of pBSC1 containing the intergenic region between *dnaA* and *dnaN* (putative *oriC* region) was subcloned into pBluescript II SK(+), resulting in pBSC2. Hybridizations with three selected probes from the *oriC* region indicated a moderate homology among the cloned *oriC* regions (data not shown).

### Comparisons of the *oriC* regions

The nucleotide sequence of the cloned putative *oriC* fragments was determined on both strands and subjected to computer analysis. The overall GC content of the analysed regions of *S. antibioticus* and *S. chrysomallus* is about 65% and 63%, respectively (Table 2), and is thus, as in the *S. lividans oriC* region, approximately 10% lower than the mean GC content of known *Streptomyces* genes. The *oriC* regions of *S. antibioticus* and *S. chrysomallus* were flanked by *dnaA* and *dnaN* which are separated by 909 and 921 bp, respectively. A search for DnaA box motifs whose sequences differ up to 2 nt from the preferred sequence (TTGTCCACA) (Zakrzewska-Czerwińska & Schrempf, 1992) allowed identification of 19 putative DnaA boxes in each analysed *oriC* region (Fig. 2). Previously, 17 DnaA boxes had been identified within the *oriC* region of *S. coelicolor* A3(2) and *S. lividans* (Zakrzewska-Czerwińska & Schrempf, 1992); the 9th and 13th DnaA boxes had not been found (Fig. 2). The position and orientation of each of the 19 deduced DnaA boxes are identical in *S. antibioticus*, *S. chrysomallus* and *S. lividans* (Fig. 2). Only the distance between DnaA boxes 9 and 10 in *S. antibioticus* is shorter (2 bp) than in *S. chrysomallus* and *S. lividans* (12 bp). Alteration of the spacing between DnaA boxes inactivates replication activity of the *E. coli* origin. It is interesting that only mutants with an insertion or deletion of 10 bp (close to a full helical turn) between the DnaA boxes R2 and R3, or R3 and R4 retain a functional *oriC* (Woelker & Messer, 1993). In contrast, point mutations in the DnaA boxes of *E. coli oriC* sequence have a comparatively slight effect on replication (Langer *et al.*, 1996). Thus, the location of the DnaA boxes with respect to the helix axis is apparently important. The short stretches of DNA flanking the individual boxes are more variable (about 60% identical) in the corresponding regions between the three *Streptomyces* species.

The 57 DnaA boxes were used to determine the consensus sequence (T/C)(T/C)(G/AC)**TCCACA** (preferred bases in bold) (Table 3). The consensus sequence of the *Streptomyces* DnaA box is more variable than in other bacteria, similar to *Mycobacterium* (Salazar *et al.*, 1996). As in other organisms, bases in positions 4 and 6 of the *Streptomyces* DnaA boxes were found to be highly conserved. C-5, A-7 and C-8 were also well conserved, in contrast to the third position which is the most variable.

Following the interaction of DnaA with DnaA boxes, a local unwinding occurs at specific AT-rich sequences, characteristic for a replication origin (Bramhill & Kornberg, 1988; Gille & Messer, 1991; Hsu *et al.*, 1994). The partially unwound *oriC* then presumably provides the entry site for the replicative helicase. In *E. coli*, three AT-rich 13-mer direct repeats are localized in the 5′ part of the *oriC* region close to the first DnaA box. *B. subtilis* DnaA-mediated unwinding occurs in an AT-rich 27-mer adjacent to the DnaA boxes that are located downstream of *dnaA* (Moriya *et al.*, 1994; Krause *et al.*, 1997). None of the three *Streptomyces oriC* regions contains the AT-rich repeats adjacent to clusters of DnaA boxes. However, each of the *Streptomyces* regions contains five

**Table 2.** Characteristics of the *Streptomyces dnaA–dnaN* intergenic region (*oriC* region)

| Species | No. of DnaA boxes | GC content (mol%) | Length of *oriC* region (bp) | Sequence similarities in the *oriC* region (%) | | |
|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 |
| 1. *S. antibioticus* | 19 | 65·2 | 909 | 100 | 60 | 59 |
| 2. *S. chrysomallus* | 19 | 63·0 | 921 | 60 | 100 | 63 |
| 3. *S. lividans* | 19 | 63·2 | 934 | 59 | 63 | 100 |

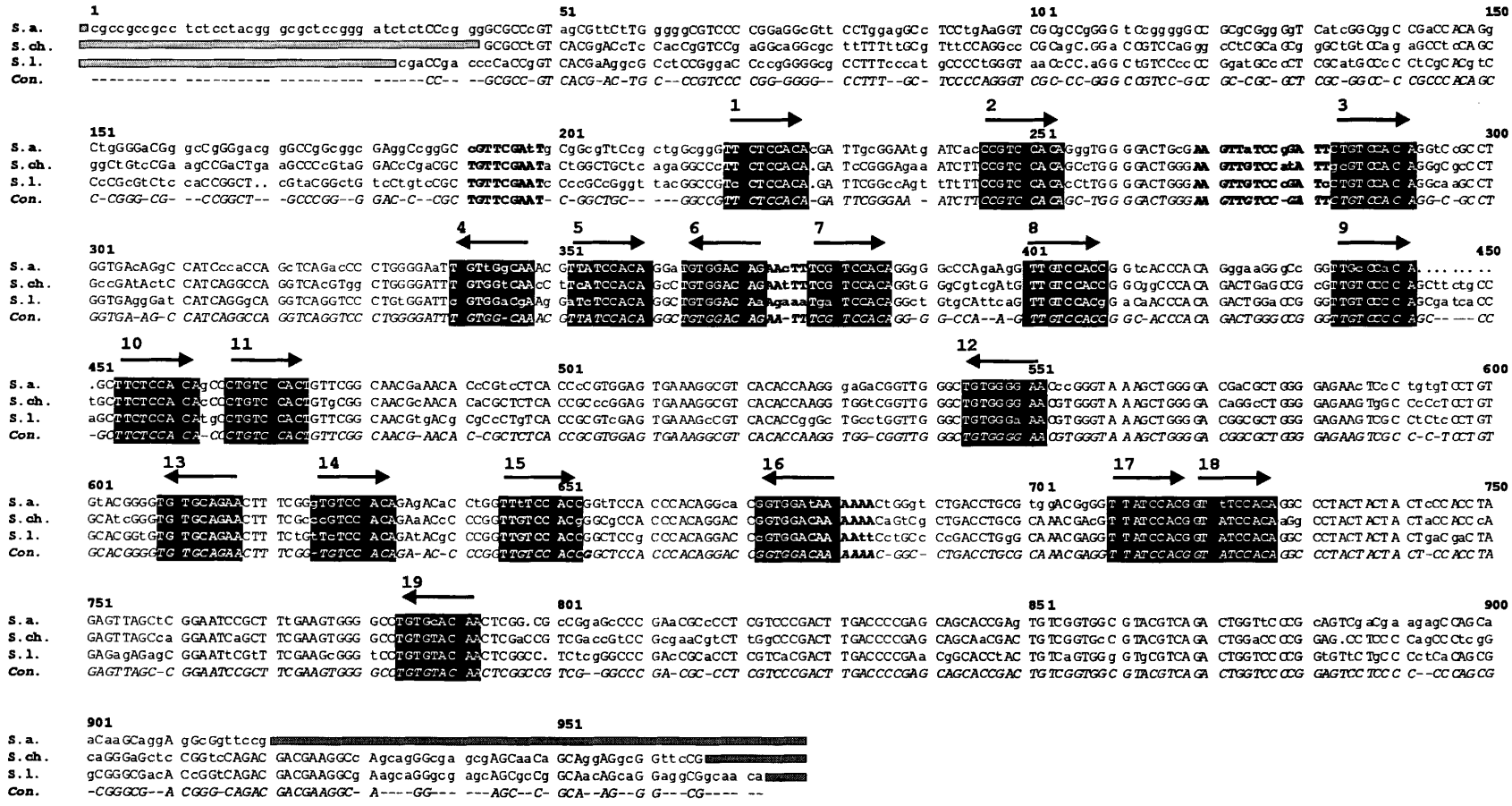


***Fig. 2.*** Alignment of nucleotide sequences of the *Streptomyces dnaA–dnaN* intergenic region (*oriC*). The positions and orientations of the DnaA boxes are indicated (black boxes and arrows). Gaps are indicated by dots. Bases which are identical in two or three of the sequences are shown in capital letters. AT-rich regions are in bold. The light grey boxes at the start of the sequences indicate the 3′ end of *dnaA*; the dark grey boxes at the end of the sequences indicate the 5′ end of *dnaN*. S.a., *S. antibioticus*; S.ch., *S. chrysomallus*; S.l., *S. lividans*; *Con.*, consensus sequence.

***Table 3.*** Frequency of nucleotide usage in *Streptomyces* DnaA boxes

| Nucleotide | No. of times used in position no.:* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| A | 1 | 0 | 9 | 1 | 2 | 1 | **55** | 0 | **41** |
| C | 11 | 10 | 13 | 5 | **51** | **56** | 2 | **57** | 6 |
| G | 5 | 1 | **32** | 0 | 4 | 0 | 0 | 0 | 7 |
| T | **40** | **46** | 3 | **51** | 0 | 0 | 0 | 0 | 3 |
| **Consensus** | | | | | | | | | |
| *Streptomyces* spp. | T/C | T/C | $^{A/}G_{/C}$ | T | C | C | A | C | A |
| *Mycobacterium* spp. | T/C | T | G/A | T | C | C | A/C | C | A |
| *E. coli* (a)† | T | T | A/T | T | N | C | A | C | A |
| *E. coli* (b)† | T | T/C | A/T | T | A/C | C | A | C/A | A |
| *E. coli* (c)† | T/C | T/C | $^{T/}A_{/C}$ | T | A/C | C | A/G | $^{A/}C_{/T}$ | A |

* Numbers/letters in bold indicate the preferred sequence.

† Consensus sequences for the *E. coli* DnaA box were determined by three different methods: (a) binding constants measurements (Schaper & Messer, 1995); (b) DNaseI footprinting analysis (Fuller *et al.*, 1984); (c) *in vivo* analysis of the effects of DnaA on transcription termination (Schaefer & Messer, 1991).

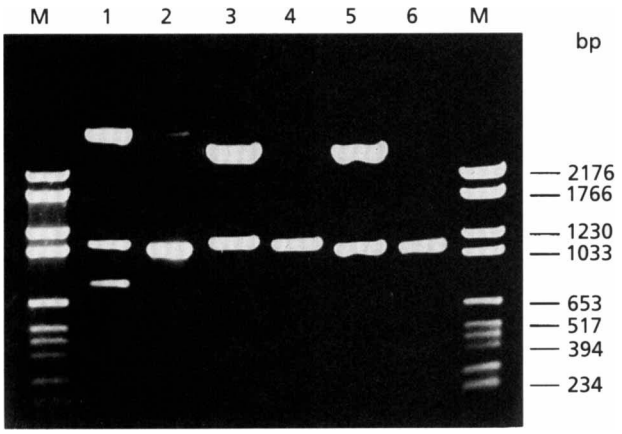


***Fig. 3.*** DNA-binding assay – selective binding of an *oriC*-containing DNA fragment using GST-DnaA(BD) beads. Plasmid DNA (1 μg) digested with *Sal*I was bound to the beads. Fragments were analysed by agarose gel electrophoresis. Lanes: 1, 3, 5, DNA loaded on the affinity column; 2, 4, 6, DNA specifically bound to the fusion protein and released from the beads by washing with 'high' salt buffer. The analysed DNA fragments were from *S. antibioticus* (lanes 1, 2), *S. chrysomallus* (3, 4) and *S. lividans* (5, 6). Lanes M, standard size markers (Boehringer VI).

short AT-rich sequences that are located close to the 5′ end of the first DnaA box (positions 191–199), between the 1st and 2nd boxes (230–245), between the 2nd and 3rd boxes (269–282), between the 6th and 7th boxes (371–378) and following the 16th box in the 3′ flanking sequence (678–684) (Fig. 3). Repeated attempts to determine DnaA-mediated unwinding of *oriC*, using permanganate footprinting, have been unsuccessful (data not presented). *S. lividans* DnaA differs from the corresponding *E. coli* protein in its acquisition of an additional stretch of 120 predominantly acidic amino acids in domain II (Majka *et al.*, 1997b). It remains to be elucidated whether the acidic domain II interacts with putative accessory basic proteins which help in the unwinding of *oriC*.

The *S. chrysomallus* minichromosome replicates in *S. lividans*. However, similar to the minichromosomes of *S. lividans*, *B. subtilis* and *Mycobacterium tuberculosis*, it is unstable and present only in low copy numbers. This apparent incompatibility could be caused by the competition of the same replicons for DnaA. Thus, as in *B. subtilis* (Moriya *et al.*, 1994), the initiation of *Streptomyces oriC* replication appears to be tightly controlled.

## Interaction of DnaA with DnaA boxes

To test whether the previously characterized *S. lividans* DnaA protein binds to the cloned *Streptomyces oriC* fragments, a recently described DNA-binding assay was performed (Majka *et al.*, 1997a). The fusion protein, consisting of the BD of DnaA and GST, was fixed to glutathione-Sepharose beads and its specific interactions with the cloned *oriC* fragments were tested (Fig. 3). Each of the cloned DNA fragments of *S. antibioticus* and *S. chrysomallus*, as well the previously characterized *oriC* of *S. lividans* (control), was selectively bound to the GST-DnaA(BD) fusion protein.

His-tagged *S. lividans* DnaA (Majka *et al.*, 1997b) was investigated for its binding properties with respect to various DnaA boxes. To evaluate the interactions of

***Table 4.*** Kinetic rate constants for the binding of DnaA to DnaA boxes

| DnaA box* | Sequence (5′–3′)† | Kinetic constants‡ | | |
|---|---|---|---|---|
| | | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (nM)§ |
| $1_{dnaA} = 6_{oriC}$ | GAGACACTTGTCCACACAACTTG | $1{\cdot}2 \times 10^6$ | $1{\cdot}4 \times 10^{-2}$ | 12 |
| $2_{dnaA}$ | GAGACACTGGGGACAACAACTTG | – | – | – |
| $2_{oriC}$ | GTTTTTTCCGTCCACACCTTGGG | $5{\cdot}0 \times 10^5$ | $3{\cdot}9 \times 10^{-2}$ | 78 |
| $4_{oriC}$ | GTGGATTCGTGGACGAAGAAATG | – | – | – |
| $5_{oriC}$ | GTGGATTATCTCCACAAGAAATG | $6{\cdot}3 \times 10^6$ | $2{\cdot}0 \times 10^{-2}$ | 32 |
| $10_{oriC}$ | CACCAGCTTCTCCACATGCCTGT | – | – | – |
| $12_{oriC}$ | GTTGGGCTGTGGGAAACGTGGT | – | – | – |
| $17_{oriC}$ | AACGAGGTTATCCACGGTATCCA | – | – | – |
| Nonsense box | TTGTGCGATATAGTTCTCCGA | – | – | – |

* The numbering of DnaA boxes is derived from the *oriC* region according to Fig. 3; the promoter region of the *S. lividans dnaA* gene contains two DnaA boxes: $1_{dnaA}$ and $2_{dnaA}$ (Zakrzewska-Czerwińska *et al.*, 1994).

† Bold letters indicate the region of specific recognition (DnaA box).

‡ –, non-specific binding ($K_D > 200$ nM).

§ $K_D = k_{off}/k_{on}$.

DnaA with individual DnaA boxes, as well as possible effects of adjacent DnaA boxes, surface plasmon resonance (for review, see Bondeson *et al.*, 1993; Malmqvist, 1993) was used. The major advantage of this technique is that protein–DNA interactions can be monitored in real time. Association and dissociation constants are subsequently recorded in the same experiment. A gel retardation assay is hampered by the need for recording a rapid reaction with a small number of data points. A technical difficulty is the lag time from loading of the sample onto the gel to the separation of free and bound reactants (Bondeson *et al.*, 1993). In contrast to surface plasmon resonance, gel retardation assays do not provide a real-time picture of the association events.

Oligonucleotides containing single DnaA boxes were synthesized (Table 4) and constructed in such a way that the DnaA box was flanked either on one side ($4_{oriC}$, $5_{oriC}$, $1_{dnaA}/6_{oriC}$, $2_{dnaA}$) or on both sides ($2_{oriC}$, $10_{oriC}$, $12_{oriC}$, $17_{oriC}$) by corresponding sequences from *oriC* or from the *dnaA* promoter. As a control for non-specific binding, a duplex oligonucleotide with a scrambled consensus sequence was included (Table 4). The oligonucleotides were then treated with varying concentrations of DnaA. The kinetic parameters for the binding of DnaA to the DnaA box were deduced from sensograms (Fig. 4 and by similar experiments) using the BIAevaluation version 2.1 program. The shape of the sensograms for the scrambled oligonucleotide was not dependent on DnaA concentration (data not shown). The curves obtained for the scrambled oligonucleotide were subtracted from the curves obtained for the oligonucleotide containing the DnaA box.

All sensograms were monophasic and could therefore be assumed to follow a pseudo-first-order kinetic model.

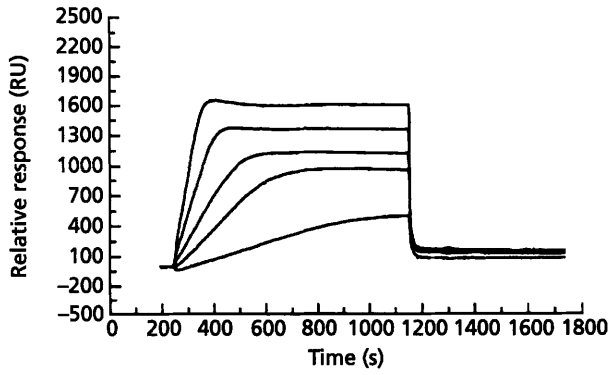


***Fig. 4.*** Plasmon surface resonance studies. Sensograms obtained at different concentrations of DnaA interacting with a DNA fragment containing DnaA box $1_{dnaA}/6_{oriC}$. The biotinylated double-stranded oligonucleotide was immobilized on a streptavidin-coated chip of the BIAcore apparatus, as described in Methods. From bottom to top, the concentration of DnaA was 17·4, 43·3, 87, 108 and 174 nM, respectively.

The apparent $k_{off}$ and $k_{on}$ rate constants were determined from the association and dissociation curves, respectively. $k_{off}$ and $k_{on}$ were calculated by fitting the data to the equations $R = R_0 e^{-k_{off}(t-t_0)}$ and $R = R_{eq}[1 - e^{-(k_{on}C + k_{off})(t-t_0)}]$ where $R_0$ is the initial response, $R_{eq}$ is the response at equilibrium and $C$ is the molar concentration of protein in solution (BIAevaluation software handbook, 1996; BIAcore). Since the response is directly proportional to the concentration of complexes formed, the response value ($R$) may be used without conversion. The $k_{off}$ and $k_{on}$ values obtained at different protein concentrations did not vary signifi-

cantly. The equilibrium dissociation constant, $K_D$, was calculated as the ratio between dissociation and association rate constants ($k_{off}/k_{on}$) (Table 4).

Only three of the eight individual DnaA boxes analysed ($2_{oriC}$, $5_{oriC}$ and $1_{dnaA}/6_{oriC}$) exhibited specific binding of DnaA; the $K_D$ varied between 12 and 78 nM (Table 4). Similar values were obtained by mobility shift assay (data not shown). The DnaA box with the 'perfect' sequence (TTGTCCACA) showed the highest affinity to DnaA (12 nM). DnaA boxes $2_{dnaA}$, $4_{oriC}$, $12_{oriC}$ and $17_{oriC}$, which differ from the consensus at highly conserved positions (4, 7 and 9), were not recognized specifically by DnaA, i.e. the $K_D$ was >200 nM. DnaA box $10_{oriC}$ was not bound specifically by DnaA, despite the fact that its sequence differs by one base from the consensus at the most variable third position. Different techniques were employed to define the consensus sequence of the *E. coli* DnaA box (Table 3). Having determined the equilibrium binding constant by gel retardation, the consensus sequence TT(A/T)-TNCCACA was stringently defined (Schaper & Messer, 1995). It corroborates the fact that the DnaA box $10_{oriC}$ (C is at the third position instead of A or T) was not specifically recognized by DnaA.

Future studies are planned to elucidate the initiation of replication of *Streptomyces oriC*, which is more complex than the corresponding process in *E. coli*.

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