A new *Myxococcus xanthus* gene cluster for the biosynthesis of the antibiotic saframycin Mx1 encoding a peptide synthetase

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The gene cluster for the biosynthesis of the heterocyclic quinone antibiotic saframycin Mx1 of *Myxococcus xanthus* DM504/15 was inactivated and tagged by Tn5 insertions. The tagged genes were cloned in *Escherichia coli* and used to select overlapping cosmid clones spanning 58 kb of the *M. xanthus* genome. Gene disruption experiments defined a ≥ 18 kb contiguous DNA region involved in saframycin biosynthesis. Sequencing of part of this region revealed a large ORF containing two 600-amino-acid domains with similarity to peptide synthetase amino-acid-activating sequences, suggesting that saframycin Mx1 is synthesized by a nonribosomal multienzyme complex, similar to other bioactive peptides.

Keywords: *Myxococcus xanthus*, saframycin Mx1, antibiotic, peptide synthetase

INTRODUCTION

Saframycins belong to the group of heterocyclic quinone antibiotics including mitomycins and streptonigrin (Nakatsubo *et al.*, 1977; Weinred *et al.*, 1982) which bind covalently to DNA and inhibit cellular DNA and RNA synthesis (Ishiguro *et al.*, 1978, 1981; Kishi *et al.*, 1984; Lown *et al.*, 1982). Saframycins A, B, C, D and E were isolated first from *Streptomyces lavendulae* (Arai *et al.*, 1977, 1980). Saframycin Mx1 is produced by the Gram-negative myxobacterium *Myxococcus xanthus* (Irschick *et al.*, 1988). Saframycins have antimicrobial (Irschick *et al.*, 1988) and good antitumour activity (Arai *et al.*, 1980; Kishi *et al.*, 1984). Fig. 1 shows the structure of saframycin Mx1 (Trowitzsch-Kienast *et al.*, 1988). The dimeric N-heterocyclic quinone which is present in all saframycins is derived from two tyrosine molecules and the side chains are derived from glycine and alanine (Mikami *et al.*, 1985).

The myxobacteria grow in soil and have a complex life cycle with multicellular differentiation. Similar to differentiating actinomycetes and bacilli, they produce a large number of secondary metabolites (Reichenbach, 1986; Reichenbach *et al.*, 1988). The knowledge of genes involved in the synthesis of myxobacterial secondary metabolites is however still limited to two gene clusters

involved in the biosynthesis of the macrolide antibiotics TA (myxovirescin A) from *M. xanthus* (Gerth *et al.*, 1982; Tolchinsky *et al.*, 1992; Varon *et al.*, 1992) and the soraphen A gene cluster of *Sorangium cellulosum*. Genes for

Fig. 1. Structure of saframycin Mx1 derived from alanine, two tyrosines and glycine.

Abbreviation: CIAP, calf intestine alkaline phosphatase.

The GenBank accession number for the sequence reported in this paper is U24657.

the latter have been cloned and sequenced, revealing repetitive type I polyketide synthetases (Schupp *et al.*, 1995; Hopwood & Khosla, 1992).

Here we report the cloning in *Escherichia coli* of 58 kb contiguous *M. xanthus* DNA involved in the biosynthesis of saframycin Mx1. Transposon mutagenesis and gene disruption by homologous recombination in *M. xanthus* were used to define a ≥ 18 kb DNA region involved in antibiotic production, and sequencing of part of this region revealed similarities to peptide synthetases, consistent with the biosynthetic origin of saframycin Mx1.

METHODS

Bacterial strains and plasmids. These are listed in Table 1. For gene disruption experiments the conjugative plasmids pSUP202 and pCIB132 were used. For DNA sequencing, fragments were cloned in pBluescript II SK(+).

Media and culture conditions. *E. coli* was grown at 37 °C in Luria broth (LB) or on LB agar. *Sarcina lutea* was grown at 30 °C on solid DST medium (Oxoid) or in liquid BHI medium (BBL). *M. xanthus* was grown at 28 °C in S45 medium (3 g casein peptone l⁻¹, 2 g MgSO₄ · 7H₂O l⁻¹, 0.5 g CaCl₂ · 2H₂O l⁻¹, 1.2 %, w/v, HEPES, pH 7.2), or on solid S46 medium (S45 with 12 g agar l⁻¹). Antibiotics were used at the following concentrations for *E. coli* and *M. xanthus*: ampicillin 50 µg ml⁻¹, kanamycin 50 µg ml⁻¹, streptomycin 100 µg ml⁻¹, tetracycline 10 µg ml⁻¹ and chloramphenicol 34 µg ml⁻¹.

Tn5 mutagenesis of *M. xanthus* DM504/15. An overnight culture of *E. coli* S17-1 containing pSUP2021 and grown under kanamycin, ampicillin and chloramphenicol selection was diluted 100-fold with LB medium without antibiotics and incubated for a further 6 h. Subsequently 1×10^{10} cells of this *E. coli* culture and 5×10^8 cells of *M. xanthus* DM504/15 (grown at 28 °C to a cell density of 2×10^8 ml⁻¹ and preincubated for 10 min at 45 °C) were mixed, sedimented (8000 g, 3 min), resuspended in 150 µl S45 medium and spotted onto S46 agar without antibiotics. After overnight incubation at 28 °C, cells were scraped off the agar and resuspended in 1 ml S45 medium. Portions (100 µl) were plated onto S46 plates containing 100 µg streptomycin ml⁻¹ (streptomycin resistance encoded by Tn5 is expressed in *M. xanthus* but not in *E. coli*; Madozier *et al.*, 1986) and 50 µg kanamycin ml⁻¹ and incubated for 6 d at 28 °C.

Biological assay (biotest) for saframycin Mx1 production. *M. xanthus* DM504/15 and its derivatives were grown for 5 d at 28 °C on S46 agar (20 ml per plate) without antibiotics. Agar plugs (3 mm diameter) with single colonies were placed onto DST agar freshly overlaid with 10 ml DST agar at 45 °C and 100 µl of an overnight culture of *Sarcina lutea*. After overnight incubation at 30 °C, the inhibition zones of *S. lutea* were measured. This biotest detects ≥ 0.5 ng saframycin Mx1. Colonies of strain DM504/15 produce 10–25 ng saframycin Mx1 and inhibition zones of 10–14 mm diameter; non-producing mutants did not inhibit growth of *S. lutea*.

Gene disruption of *M. xanthus* DM504/15. pCIB132 derivatives were introduced by conjugation into *M. xanthus* DM504/15 using the same procedure as for the Tn5 mutagenesis. Kanamycin-resistant transconjugants containing plasmids integrated into the genome via homologous recombination were selected.

DNA manipulations. Standard genetic techniques for *in vitro* DNA manipulations and cloning were used (Sambrook *et al.*,

1989). Genomic DNA preparations were performed as described by Neumann *et al.* (1992). For Southern blots and colony hybridizations the digoxigenin labelling and detection kit from Boehringer-Mannheim and Hybond N-Nylon-membranes from Amersham were used.

Construction of *M. xanthus* DM504/15 genomic libraries. *M. xanthus* DM504/15 total DNA was partially digested with *Sau3AI* and size-fractionated in 15–40 % (w/v) sucrose gradients. Fractions containing DNA fragments of 23–42 kb were pooled and ligated to pWE15 digested with *Bam*HI and treated with calf intestine alkaline phosphatase (CIAP). The ligation mixture was packaged *in vitro* using Gigapack Plus (Stratagene).

DNA sequencing. Double-stranded DNA fragments were cloned into pBluescript SK(+) and sequenced by the dideoxy nucleotide chain-termination method (Sanger *et al.*, 1977) with dye-labelled primers and an Applied Biosystems Automated Sequencer (model 373A). Universal primer and oligonucleotide primers designed according to the newly obtained DNA sequences were used.

Computer analysis of DNA and protein sequences. The DNA sequences were assembled and analysed using software Version 1.20 from Applied Biosystems and the UWGCG program package version 8.0 (Devereux *et al.*, 1984).

HPLC analysis of saframycin Mx1 production. S45 medium (20 ml) was inoculated with 1 ml cells from a –70 °C stock in 20 % (v/v) glycerol and incubated with shaking for 3 d at 28 °C. Subsequently a second preculture in 20 ml S45 medium was inoculated with 5 % (v/v) of the first culture and incubated with shaking for 3 d at 28 °C. Then 1.5 ml of the second culture was transferred to a 200 ml Erlenmeyer flask containing 50 ml Saf3 medium [casein peptone, 3 g l⁻¹; dextrin A-332, 32 g l⁻¹; MgSO₄ · 7H₂O, 2 g l⁻¹; CaCl₂ · 2H₂O, 0.5 g l⁻¹; sodium citrate, 4 g l⁻¹; absorber resin XAD 1180 (Rohm and Hass, Frankfurt, Germany), 50 g l⁻¹; pH 7.2]. After 3 d shaking at 28 °C, 5 ml 5 % (w/v) casitone was added. After additional incubation for 3 d at 28 °C the absorber resin XAD 1180 was harvested on a polyester sieve (Sartorius, B 420-47-N) and eluted with 20 ml 90 % (v/v) 2-propanol by shaking for 30 min in 50 ml Falcon tubes at room temperature. The 2-propanol eluate was centrifuged for 5 min and the clear supernatant was analysed by HPLC for its saframycin Mx1 content. Reverse-phase HPLC analysis was performed by using a silica-based stationary phase [Lichrospher 60 A RP-18 (5 µm), Merck] in a 12.5 cm column and a phosphate buffer (pH 3) acetonitrile gradient as mobile phase. Saframycin Mx1 was detected by absorption at 260 nm. Typical yields for strain DM504/15 were 18–24 mg (1 culture)⁻¹. The detection limit was 3 mg l⁻¹.

RESULTS

Tn5 mutagenesis of *M. xanthus* DM504/15 and isolation of saframycin Mx1 non-producers

Tn5 and its derivatives have been used to mutagenize *M. xanthus* and to identify and clone a number of genes (Kroos & Kaiser, 1984; Kroos *et al.*, 1986; Kuner & Kaiser, 1981). Transposition of Tn5 into the genome occurs after conjugative transfer of the suicide plasmid pSUP2021 from *E. coli* to *M. xanthus* (Saulnier *et al.*, 1988). For the saframycin production strain DM504/15, this technique yielded kanamycin- and streptomycin-resistant mutants at a frequency of about 1×10^{-5} per recipient cell. Southern blots using genomic DNA from

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics*	Source or reference
<i>E. coli</i>		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1</i>	Stratagene Cloning Systems
	<i>hsdR17 supE44 relA1 lac [F'</i>	
	<i>proAB lacI^q lacZΔM15 Tn10 (Tc^r)</i>	
XL1-Blue MR	<i>recA1 endA1 gyrA96 thi-1</i>	Simon <i>et al.</i> (1983, 1986)
	<i>supE44 relA1 lac Δ(mcrA)183,</i>	
	<i>Δ(mcrCB-hsdSMR-mrr)173</i>	
S17-1	<i>recA pro thi hsdR chr::RP4-2</i>	
<i>M. xanthus</i>		
Mx x48	Saf ⁺ , wild-type	Irschick <i>et al.</i> (1988)
DM504/15	Saframycin Mx1 production strain, aobtained by UV mutagenesis of <i>M. xanthus</i> Mx x48	This study
AP66/254	DM504/15::Tn5, Saf ⁻ Km ^r Sm ^r	This study, Fig. 4
AP70/9-1	DM504/15::Tn5, Saf ⁻ Km ^r Sm ^r	
<i>Sarcina lutea</i>		
ATCC 9341	Saframycin Mx1 sensitive	American Type Culture Collection
Plasmids		
pSUP2021	Ap ^r Cm ^r Tn5 (Km ^r Sm ^r) <i>oriT</i> (RK2)	Simon <i>et al.</i> (1983, 1986)
pSUP202	Ap ^r , Cm ^r , <i>oriT</i> (RK2)	
pCIB132†	Ap ^r Cm ^r Km ^r <i>oriT</i> (RK2)	J. M. Ligon, personal communication
pBluescript II SK(+)	Ap ^r	Stratagene Cloning Systems
pWE15	Ap ^r Km ^r , <i>E. coli</i> cosmid vector	
pAP78/1	16 kb <i>EcoRI</i> fragment of AP66/254 in pSUP202	This study
pAP78/5	8.8 kb <i>EcoRI</i> fragment of AP70/9-1 in pSUP202	
pAP93/8	Cosmid clone of <i>M. xanthus</i> DM504/15 in pWE15 with an insert of 42 kb	
pAP101/19	Cosmid clone of <i>M. xanthus</i> DM504/15 in pWE15 with an insert of 38 kb	This study, Fig. 4
pAP108/3	Cosmid clone of <i>M. xanthus</i> DM504/15 in pWE15 with an insert of 38 kb	
pAP98/16	2.4 kb <i>Bam</i> HI fragment of pAP93/8 in pCIB132	
pAP98/17	1.6 kb <i>Bam</i> HI fragment of pAP93/8 in pCIB132	
pAP98/22	0.8 kb <i>Bam</i> HI fragment of pAP93/8 in pCIB132	
pAP98/23	0.9 kb <i>Bam</i> HI fragment of pAP93/8 in pCIB132	
pAP98/1	3 kb <i>EcoRI</i> fragment of pAP93/8 in pCIB132	
pAP103/3	6 kb <i>EcoRI</i> fragment of pAP101/19 in pCIB132	
pAP110/2	7 kb <i>EcoRI</i> fragment of pAP108/3 in pCIB132	
pAP110/11	16 kb <i>EcoRI</i> fragment of pAP108/3 in pCIB132	
pAP113/3	8 kb <i>EcoRI/EcoRV</i> fragment of pAP108/3 in pCIB132	

*Saf⁺/-, saframycin Mx1 production/non-production.†Derivative of pSUP2021 constructed by reversal of the 5 kb *NotI* fragment, followed by removal of the resulting 3 kb *Bam*HI fragment, leaving a unique *Bam*HI cloning site.

Fig. 2. Southern blot probed with the labelled 3.4 kb *Hind*III fragment of Tn5. Lanes: 1, AP66/254; 2, pAP78/1; 3, AP70/9-1; 4, pAP78/5; 5, genomic DNA of the wild-type strain DM504/15. Genomic and plasmid DNAs were digested with *Eco*RI. Size markers are indicated in kb.

25 independent Tn5 insertion mutants indicated that the transposon integrated at different sites (data not shown).

In four separate experiments, 2160 Tn5 integrative mutants of *M. xanthus* DM504/15 were generated and screened for saframycin Mx1 production using the biological assay. A total of 27 (1.25 %) saframycin Mx1 non-producers were found and confirmed by HPLC analysis.

Gene disruption with Tn5-containing DNA fragments derived from saframycin Mx1 non-producers

Genomic DNA of Tn5-containing saframycin Mx1 non-producing mutants AP66/254 and AP70/9-1 was digested with *Eco*RI (does not cut Tn5, 5.7 kb), ligated to the *Eco*RI-digested and CIAP-treated conjugative vector pSUP202 and transformed into *E. coli* S17-1. Two kanamycin-resistant clones were analysed: pAP78/1, derived from AP66/254, contains a 16 kb *Eco*RI fragment; pAP78/5, derived from AP70/9-1, contains an 8.8 kb *Eco*RI fragment. Southern blot analysis with a Tn5-specific probe showed that the cloned fragments had the same size as the genomic fragments (Fig. 2). Chromosomal DNA of *M. xanthus* DM504/15 showed no cross-hybridization with Tn5.

The role of these *Eco*RI fragments in saframycin Mx1 biosynthesis was confirmed by gene disruption in strain *M. xanthus* DM504/15. Both plasmids gave kanamycin-resistant transconjugants, with frequencies of 1.4×10^{-3} for pAP78/1 and 1.4×10^{-4} for pAP78/5. Saframycin Mx1 production determined by the biotest was abolished in 148/153 (pAP78/1) and in 98/176 (pAP78/5; smaller clone) transconjugants. Southern blot analysis showed that the saframycin non-producing transconjugants contained the plasmids integrated by a single crossover in the genome while in the kanamycin-resistant producer strains

Fig. 3. Southern blots with genomic DNA of the wild-type DM504/15 (lane 1) and cosmid pAP93/8 (lane 2). Total genomic DNA and cosmid DNA were digested with *Eco*RI and probed with (a) two labelled *Eco*RI/*Hind*III fragments of 10 and 2.6 kb from pAP78/1, and (b) two labelled *Eco*RI/*Hind*III fragments of 1.9 and 3.5 kb from pAP78/5. The 9 kb fragment in lane 2 is due to cross-hybridization with vector DNA. Size markers are indicated in kb.

Tn5 had transposed to random sites in the genome (data not shown).

Results obtained in the biotest were confirmed by HPLC analysis for three producing and three non-producing transconjugants from each plasmid: the six strains containing random Tn5 insertions produced between 20 and 24 mg saframycin Mx1 l⁻¹; this is similar to the productivity of the parental strain DM504/15, which produces between 18 and 24 mg saframycin Mx1 l⁻¹. The transconjugants in which the plasmids had integrated by homologous recombination produced no saframycin. These results demonstrated a direct correlation between the cloned fragments in pAP78/1 and pAP78/5 and saframycin Mx1 production.

Cloning of the saframycin Mx1 biosynthesis genes from *M. xanthus* DM504/15

A cosmid library of *M. xanthus* DM504/15 DNA in pWE15 was screened with DNA probes derived from the *Eco*RI inserts of pAP78/1 and pAP78/5. To avoid cross-hybridization between the kanamycin-resistance gene of Tn5 and the kanamycin-resistance gene of the vector, the central part of Tn5 was deleted from the *Eco*RI fragments by digestion with *Hind*III. As a result two *Eco*RI/*Hind*III fragments of 10 and 2.6 kb for pAP78/1 (probe A) and two *Eco*RI/*Hind*III fragments of 1.9 and 3.5 kb for pAP78/5 (probe B) were obtained. In a first screening, 2500 colonies were analysed with probe A and probe B.

Fig. 4. The saframycin Mx1 biosynthesis locus of *M. xanthus* DM504/15. The cosmids are shown on top. E, *EcoRI*; E*, *EcoRI* sites of the vector; EV, *EcoRV*. Filled triangles indicate the positions of the Tn5 insertions in the non-producing strains AP70/9-1 and AP66/254. The hatched box represents the sequenced region. The bars below show the DNA fragments that were cloned into pCIB132 and used for gene disruption experiments; black bars indicate clones whose insertion into the DM504/15 genome abolished saframycin production; white bars indicate no effect on saframycin production. The numbers in parentheses indicate how many independent clones were analysed for antibiotic production. The arrows at the bottom of the figure indicate the minimal possible size of the *saf* transcription unit deduced from the gene disruption experiments.

One cosmid clone, pAP93/8 (see Fig. 4), hybridized with both DNA probes (Fig. 3a, b), indicating that the sites of Tn5 insertions in AP66/254 and AP70/9-1 are close to each other (Fig. 4).

As expected, the chromosomal *EcoRI* fragments of *M. xanthus* DM504/15 detected by probes A and B are about 6 kb (size of Tn5) smaller than the inserts in pAP78/1 and pAP78/5 (Fig. 3). The *EcoRI* fragments from the cosmid pAP93/8 are of the same size as the chromosomal fragments, indicating that the complete wild-type fragments have been cloned. Additional screening identified the cosmids pAP101/19 and pAP108/3 (Fig. 4). Together the cosmids pAP93/8 and pAP108/3 cover about 58 kb. Fig. 4 shows restriction maps of the cosmids and of the genomic region they represent.

Size of the region involved in saframycin Mx1 biosynthesis

DNA fragments of the three cosmids pAP93/8, pAP101/19 and pAP108/3 were used for gene disruption experiments. Four *Bam*HI and four *Eco*RI fragments were subcloned into the conjugative plasmid pCIB132 and transformed into the *E. coli* donor strain S17-1 (Fig. 4). All fragments gave kanamycin-resistant (encoded by pCIB132) *M. xanthus* DM504/15 transconjugants, presumably by homologous recombination, with frequencies ranging between 1.4×10^{-3} and 1.5×10^{-6} . The saframycin Mx1 productivity of these transconjugants was determined with the biotest. The six fragments represented by black bars in Fig. 4 abolished biosynthesis of saframycin Mx1 after integration. Open bars represent

fragments whose integration had no effect on antibiotic production and therefore most probably contain the beginning and the end of the saframycin Mx1 biosynthetic gene cluster. To determine the position on the left end of the gene cluster more precisely, plasmid pAP113/3 (Fig. 4) was constructed by a partial *Eco*RI digestion of pAP110/11 followed by digestion with *Eco*RV, infilling and subsequent ligation. This plasmid carries an 8 kb insert and gave saframycin-Mx1-positive transconjugants. Thus the left end of the gene cluster is located within this 2 kb region. Based on these gene disruption experiments, and on the positions of the Tn5 integration sites, the size of the region involved in saframycin Mx1 biosynthesis is at least 18 kb (Fig. 4).

Sequence analysis

The nucleotide sequence (Fig. 5) of 5809 bp of the saframycin Mx1 biosynthetic gene cluster was determined. It has an overall G + C content of 71 mol %, typical for myxobacteria. Analysis of the sequence with the program CODONPREFERENCE (Devereux *et al.*, 1984) revealed one probably translated ORF (Fig. 5). The ORF (*safA*) has more than 1936 amino acids and its start point and stop codon are outside the sequenced region. Sequence comparison using TFASTA (Devereux *et al.*, 1984) revealed an overall identity of the deduced amino acid sequence of *safA* of about 34 % compared to diverse peptide synthetases, for example: gramicidin S synthetase II from *Bacillus brevis* (Krättschmar *et al.*, 1989; Turgay *et al.*, 1992); surfactin synthetase from *B. subtilis* (Cosmina *et al.*, 1993); pyoverdine synthetase D from *Pseudomonas aeruginosa* (T. R. Merrimann and others, unpublished,

Fig. 5. For legend see facing page.

3001 ATTCGGGAGGTGCTCGCCACGTATGGCGCCCTCCTGCGGGGCGAGCAGTCCGCGGCGTCTCCGTGTCGCGCTACGCCGAGTACGCCGCGCGGAGCAGG
 I R E V L A T Y G A L L R G E Q S A A S V S A Y A E Y A A R E Q A
 3101 CCTGGCTGAGCGGGCCGGTGTGTGGCCAGCAGCTCGAACTGGCGCCGGAAGCTCACGGACCTGCCCTGCTGCGGCTCTTACCGATCGGCCGCGTCC
 W L S G P V C G Q Q L E H W R R K L T D L P L L R L F T D R P R P
 3201 CGTCATCAAGACGACCGCTGCGACTCAGTCTCCACCCCTGCTGCGGCTCGAGCTGTGCGGCGGATGGAAGCGCTGGCCTCGCGAACGGGGCCTGCGC
 V I K T H R C D S V S T L L P L E L S R R M E A L A S R N G G L R
 3301 GCGGCGGTGCTGGCGGCTTTAGGTGCTGCTGCTCCCGTACACCGGGCAATCCGATGTGACAGTGGGCGTTCGCGCGGACCTCCGGAGCGAAGCGAAC
 A A V L A G F Q V L L S R Y T G Q S D V T V G V R A D L R S E A E R
 3401 GAGGCGGCTCTCGGGCCCGCAGCACCGCCTGGTCTGCGGACCGACCTGCGCGGCGAGCCGCTTCATGGAGACGCTCCTGCGCGTCGACGTGGC
 G R L L G P A S T A L V L R T D L R G E P S F M E T L L R V D V A
 3501 CCGCAGGAGGCGGAGCGGTTCGCCAGCGTGCCTTCGTCAGCTGGTGACACGCTGAGCCGCGCCGTGACCTGAGCCGACCGCCCTCTTTTCAAGTG
 C E E A E R F A S V P F G C T L V D T L Q P A R D L S R T P F F Q V
 3601 ATGTACCTGTTTTCGGATGCGCGGAGGAGCTGGCGGTGGCGCCGCGCTGCGCGCCCTGGCGCAGGCGTTCGCGTTCGCGACCAACCCCGGTGGACATCA
 M Y L F R D A R E E L A V G A G L R A L A Q A L P F G T T P V D I T
 3701 CCCTGGCGGCGACCGCCACCGAGCAGGCGCTCCACCTGCGCGTCACTTCACTCCGACCTGTACGACGCGAGCACCGCGCGCGCTGTGGGCCACCT
 L A A T A T E Q G L H L R V D F N S D L Y D A S T A R R L L G H L
 3801 GCGCAGCTGCTGTCGCGGCGGTGGAGCTCCGGGGAAGTCCATCGCCCGGTGCGCTGCTGCGCGAGGAGGAGTGGAGGAGGCTCTTCGCGACTGG
 R T L L S A A V D A P G K S I A R L P L L A E E E W R Q V F G D W
 3901 AACCAGGCGGAGCAGCCCTCCCGTGGCGTGTGTCAGGAGCTCGTCAGGAGCAGGCGCGCGCACCCCGATGCCGTGGCGGTACGCTGCGAGGGCC
 N Q A E H A L P S R C L H E L V E E Q A A R T P H A V A V T C E G H
 4001 ACCAGCTCACCTACGCGGAGCTGGATGCGCACGCCAACAGCTCGCCCTACCTGCGCGAGCTCGGGCTGGCTCCGGAAGGTGGGGTGGCAGCTCTGCCT
 Q L T Y A E L D A H A N Q L A H L R Q L G L A P E G R V A I C L
 4101 GGAGCGGTCCGTGGAGATGGTACCGCCATGCTGGCGGTGCTCAAGGCGCGGTGCTACGTCGCGTGGATCCGAGTACCCACCGAGCGCTGGCG
 E R S V E M V T A M L A V L K A G G A Y V P L D P E Y P T E R L A
 4201 CAGCTGTTGCGGAGTGGCGGCGCAGTGGTGTCTCAGCAGCAGTCCCTGCGGACCGGGTGAAGATGGTGGCGCGCGCGCGTGTTCGTGGACACGG
 Q L F A D C E A H V V L T Q Q S L A D R V K M V A A R R V F V D T D
 4301 ACTGGCGGTTCATCGCGCGTCCCGCGGCGAGCGCGCCCGCGGTGCTCAGCGCGGACCACCTCGCCTATCTCATCTACCTCGGGGTCCACCGGCAC
 W P V I A A C P R Q A P P P V V T P D H L A Y L I Y T S G S T G T
 4401 GCCCAGGCGGTGATGCTCTCTCACCGCGCATCGTGAACAATCTCGCGTGGCGTCAGCGCACCTGGCGGTGTGTCGCGGAGGACCGGGTGTGTCAGAAC
 P K A V M L S H R G I V N N L A W R Q R T W P L S A E D R V L Q N
 4501 CACTCCTTCAGCTTCGACCCGTCGGTGTGGGCCACCTTCTGGCGCTCCTGGTGGGGGCCAGAGCGGTGCTCACCCCGTCCGGCCAGCACTACGACAGCA
 H S F S F D P S V W A T F W P L L V G C A R A V L T P S G C S K
 4601 AGCGTGGTGGGCGTGTGCGTGAACAGGGCATCACCGTGTATGGCGGCTGCGCTCAAGCGCGTGTGATGGAGGAGCGTGGATGGCCGGTG
 A L L T V Y G A G S P L N A V L M E E P E I G R C
 4701 CACCCACCTGCGGTACGTGCTGAGCGGCGCGAGGCCCTCACTGGCGGCTGACGCGCGCATCTTCTGCGCGTCTCGCGCGCGTCCGCAACCTCTAC
 T H L R Y V L S G A E A L T G G L Q R G I F S R V S A A V A N L Y
 4801 GGCCCGACGAGACCACTCGACGCCCGCATGGAAGTGGCGCGGTGGATGCCCGGAGGACGCTCCCATCGGTGGCCAAATCGGCAACCTGCGGA
 G P T E T T I D A T A W N C P R V D A P E D A P I G R P I A N L R M
 4901 TGTACGTGCTCGACGAGCACCTCCAGCGGTTCGGGTGGTGTTCGGGTGAACCTCTGTCGCGCGGCTGGGCTGGCGCGTGGCTATCACGCCAGGCC
 Y V L D E H L Q P V P V G V P G E L F V G G V G L A R G Y H A R P
 5001 CGGCTGACTTCTCAGCGCTTCTTCCCGATCCATTCTCGAGTGTGCGGCGCGCGGTGTACCGGACGGGCGACCTCGGGCGCTACCGGGCGGACGA
 G L T S Q R F L P D P F S S D A G A R L Y R T G D L G R Y R A D G
 5101 GCCATCATGTTCTCCGCGCGGTGGACGAACAGGTCAAGGTGAGCGGCTACCGCGTGAAGTTCGAGACCGCGTGGGGCGGCGACCCCGAGC
 A I M F L G R V D E Q V K V S G Y R V E L G E V E T A L G R H P D V
 5201 TGGCGAGGCCATCGTCTGCGCGGTGAGGCGCTGACGGCATCAAGCGGTGCTGGCTTACGTACGCGCGCCAAAGGGGGCACCGGAGGCTCGCTC
 R E A I V V A R E G L Q G I K R L V A Y V T P A K G G T P E A R S
 5301 GCTCAGGCATTCTCGAGAAGATTCTGCGCGGTACATGATTCCGCGGTGTTCTGTCATCGTCAACGAGCTGCGGAAGATGCCAGCGGCAAGGTCAAC
 L T A F L E K I L P A Y M I P P V F V I V N E L P K M P S G K V N
 5401 CGCAATGCCCTGCTGCGCCGAGATGGACCGGCGGACCGCGGGCGCTACGTGCTCCGCGCACGCCCTGGAGGATGAGATTGCTCGGCCCTTCG
 R N A L P A P Q M D R P D T A G A Y V A P R T P L E D E I A S A F A
 5501 CGGGCGTGTGCGCATGACCGCGTGGGAGTGGAGGACGACTCTTTGAAGTGGGCGGACCTGCTCTGCTGGCGCGGTTCGCTCACGGCTGCTGAA
 G V L G M D R V G V E D D F F E V G G T S L L L A R L A S R L L N
 5601 CCGCTTCCAGATTGCCATCCCGGTGACACAGTCTTCAAGATTCCACGGTGGCTGGGTGGGAACTGGTGGAGACTTACCAGCGCGAGGGCCTTGAT
 R F Q I A I P V H Q F F K I P T V A G V A N V V E T Y Q R E G L D
 5701 GCCGTGCTGATGAACAGCATGCCACGGGTGGACGCGGCGCTCGCTGGCGCGGACATCTCCCGAAGGGTGGCGCTGGCGAACTACCTCGCCC
 A V L M N Q H A T R L D A D A S L A P D I S P E G L P L A N Y L A P
 5801 CGTCTCCG
 S S

Fig. 5. Nucleotide sequence of 5809 kb from the saframycin Mx1 biosynthetic region of *M. xanthus* DM504/15. The deduced amino acid sequence of the ORF is given below the DNA sequence in single-letter code. Regions containing core sequences of amino-acid-activating domains are overlined.

Fig. 6. Amino acid sequence comparisons of the deduced SafA protein and an amino-acid-activating domain (amino acids 400–1100) of the GrsB protein (Turgay *et al.*, 1992) of *B. brevis*. A window of 30 and a stringency of 16 were used with the programs COMPARE and DOTPLOT version 8.0 of the UWGCG package.

aa SafA1/A2	272/1380	343/1454	530/1647
core sequences	↓ LKAGGA	↓ SGTG	↓ GELCIGG
SafA1	--VLKAGGAYVFPDP--(X) 58--QLAYVLYTSGSSGRPKGVMVSH--(X) 165--GLAGELFIGGSGVARGYWG--		
SafA2	--VLKAGGAYVPLDP--(X) 61--HLAYLIYTSGSTGTPKAVMLSH--(X) 171--GVPGLFVGGVGLARGYHA--		
TycA	--VLKAGGAYVPIDI--(X) 61--DLAYVIYTSGETTGKPKGTMLEH--(X) 165--ADEGELCIGGGLARGYWN--		
GrsA	--VLKAGGAYVPIDI--(X) 61--DLAYVIYTSGETTGKPKGTMLEH--(X) 165--GEAGELCIGGGLARGYWK--		
GrsB-A	--ILKAGGAFVPIDP--(X) 56--DLFYIYTSGETTGKHKRVMLEH--(X) 174--GIVGELYISGANVGRGYLN--		
SrfA	--ILKAGGAYVPIDP--(X) 56--NLAYIYTSGETTGKPKGMIEH--(X) 179--GVAGELYIAGDGVGRGYLH--		
EntF	--IVEAGAAAYLPIDT--(X) 58--HTAYIIFTSGSTORPKGVMVGQ--(X) 179--GVAGDLYLTGLQLAQGLYG--		

aa SafA1/A2	571/1688	RIELGEIE	729/1842
core sequences	↓ TGD		↓ LGGHS
SafA1	-(X) 22--YRTGDLVRRVDEGELEFIGRADHQVKLRGYRIELAEIE--(X) 120--VGVKDDFFELGGSNLLA--		
SafA2	-(X) 22--YRTGDLGRYRADGAIMFLGRVDEQVKVSGYRVELGEVE--(X) 116--VGVEDDFFEVGGTSLLL--		
TycA	-(X) 20--YRTGDLAKWLTDTIEFLGRIDHQVKIRGHRIELGEIE--(X) 114--IGIRDNFYSLGGDSIQA--		
GrsA	-(X) 20--YRTGDQARWLSDNIEYLGRIDNQVKIRGHRAVELEEVE--(X) 113--IGIKDNFYALGGDSIKA--		
GrsB-A	-(X) 20--YRTGDLARWLPDGNIEFLGRADHQVKIRKHRIELGEIE--(X) 113--IGIQDNFFSLGGHSLKA--		
SrfA	-(X) 20--YRTGDVVRWLPDGTIEYLGREDQVKVRGYRIELGEIE--(X) 115--AGIFDNFFETGGHSLKA--		
EntF	-(X) 20--YRTGDVARWLDNGAVEYLGREDDQLKIRGQRIELGEID--(X) 113--QDADADFFALGGHSLLA--		

Fig. 7. Conserved amino acid sequences in the SafA domains 1 and 2 and their comparison with presumably homologous sequences from other organisms. GrsA, gramicidin S synthetase I of *B. brevis* (Hori *et al.*, 1989); GrsB-A, gramicidin S synthetase II of *B. brevis* (Turgay *et al.*, 1992); EntF, serine-activating enzyme in enterobactin synthesis (Rusnak *et al.*, 1991); TycA, phenylalanine-activating enzyme in tyrocidine biosynthesis (Weckermann *et al.*, 1988); SrfA, surfactin synthetase (Cosmina *et al.*, 1993). Perfectly conserved amino acids are printed bold. Numbers at the top indicate the positions of the amino acids (aa) within SafA. Numbers between the amino acids indicate the distances between these sequences. The core sequences were defined by Marahiel (1992).

EMBL accession number U07359). A COMPARE-DOTPLOT-analysis (Devereux *et al.*, 1984) of the amino acid sequence of SafA with itself revealed two similar domains of 43 % identity, SafA1 (approximately amino acids 200–800) and SafA2 (approximately amino acids 1300–1900), of about 600 amino acids and spaced by about 500 amino acids. SafA1 and SafA2 show 35 % identity and 59 % similarity to amino-acid-activating domains of known peptide synthetases. Fig. 6 shows a DOTPLOT comparison with the gramicidin S synthetase II from *Bacillus brevis*. Peptide synthetases are multifunctional enzymes, employing the thiotemplate mechanism (Kleinkauf & von Döhren, 1990), harbouring domains of about 600 amino acids, containing at least six highly conserved core sequences (Marahiel, 1992). Fig. 7 shows a comparison of SafA1 and SafA2 with different amino-acid-activating domains of peptide synthetases. The core sequences LKAGGA, SGTG, GELCIGG, TGD, RIELGEIE and LGGHS,

as well as the spacing between them, are well conserved in SafA1 and SafA2.

DISCUSSION

Two saframycin non-producing mutants of *M. xanthus* DM504/15 were isolated after Tn5 mutagenesis. Cloning of the Tn5-containing DNA fragments from these mutants and their integration by homologous recombination (single crossover insertion of suicide plasmids introduced by interspecific RP4-mediated conjugation) into the producer strain DM504/15 (Fig. 4) again gave non-producing mutants. This proved that antibiotic non-production was caused by the insertions and not by spontaneously occurring secondary mutations, which are common in some systems (T. Kieser, personal communication).

Cosmid cloning and restriction mapping of the Tn5-containing DNA regions showed that the sites of transposon insertion were about 9 kb apart in the DM504/15 genome (Fig. 4), suggesting that they are located within a specific saframycin biosynthesis gene cluster. Another possibility would have been that global activators of antibiotic biosynthesis (Adamidis *et al.*, 1990, 1992; Lawlor *et al.*, 1988), which tend to be scattered in bacterial genomes, could have been inactivated.

The region containing the non-mutated saframycin biosynthetic gene cluster was cloned in *E. coli* on three overlapping cosmids (Fig. 4) spanning 58 kb of DNA. Within this region, saframycin production genes were located by gene disruption in a central region of at least 18 kb. The upper limit of the size of the gene cluster cannot be deduced from these experiments because only integration of fragments internal to transcription units is mutagenic.

Sequencing of 5.8 kb of this region (Fig. 5) revealed extensive similarity to known peptide synthetases (Figs 6 and 7), providing virtual proof that the gene cluster contains structural genes for saframycin Mx1 biosynthesis. Within the deduced amino acid sequence of SafA, two peptide synthetase modules, SafA1 and SafA2 (Fig. 6), were identified on a single ORF, a common feature of peptide synthetases (Cosmina *et al.*, 1993; Smith *et al.*, 1990; Turgay *et al.*, 1992). Known active sites and core regions of peptide synthetases (Fig. 7) are conserved in SafA1 and SafA2, suggesting that they may both be active and a part of a giant protein. The biological significance of the first core (LKAGGA) is unknown, but the SGTGXPKE (Gocht & Marahiel, 1994), the TGD (Gocht & Marahiel, 1994) and the KIRGXRIEL (Pavela-Vrancic *et al.*, 1994; Tohika *et al.*, 1993) core sequences could be assigned to ATP binding and hydrolysis. The serine of the core sequence LGGXS could be shown to be the site of thioester formation (D'Souza *et al.*, 1993; Schlumbohm *et al.*, 1991; Vollenbroich *et al.*, 1993) and 4'-phosphopantetheine binding (Stein *et al.*, 1994). These findings, together with the fact that saframycin Mx1 (Fig. 1) is synthesized from amino acids (Trowitzsch-Kienast *et al.*, 1988), support the hypothesis that non-ribosomal peptide bond formation via the thiotemplate mechanism (Kleinkauf & von Döhren, 1990) is involved in the biosynthetic pathway of saframycin Mx1 and that *safA* encodes the corresponding peptide synthetase. According to this mechanism, amino acids are activated as aminoacyl-adenylates by ATP hydrolysis and subsequently covalently bound to the enzyme via carboxyl-thioester linkages. Then, in further steps, transpeptidation and peptide bond formation occur.

It will be necessary to sequence the full length of *safA*, and further genes involved in saframycin Mx1 production, to achieve a precise understanding of the saframycin biosynthetic operon. Nevertheless, the results presented in this study are consistent with findings about antibiotic genes in other myxobacteria, which are arranged as clusters (Schupp *et al.*, 1995; Tolchinsky *et al.*, 1992; Varon *et al.*, 1992), as well as in other systems (reviewed

by Hopwood & Khosla, 1992). In addition, the genes characterized are similar in their sequence and organization to other antibiotic biosynthesis genes (Cosmina *et al.*, 1993; Rusnak *et al.*, 1991; Turgay *et al.*, 1992). The cloning and characterization of biosynthetic genes for peptide antibiotics produced by myxobacteria is a prerequisite for the understanding of the regulatory mechanisms that govern expression of these secondary metabolites and will provide further insight into the regulation of saframycin Mx1 production in *M. xanthus*.

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