MOLECULAR CLONING AND CHARACTERIZATION OF THE GENE CONFERRING CURROMYCIN RESISTANCE ON A CURROMYCIN NON-PRODUCING MUTANT DERIVED FROM *STREPTOMYCES HYGROSCOPICUS* 358AV2

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We cloned six different DNA fragments from a curromycin producing strain, *Streptomyces hygroscopicus* 358AV2, which confer curromycin-resistance on a curromycin non-producing and sensitive strain, *S. hygroscopicus* RgII, a protoplast regenerant of the strain 358AV2. We studied the plasmid pSHR2 carrying one of the DNA fragments. By Southern blot analysis, the cloned DNA sequence in pSHR2 was found to be deleted in the RgII genome. From the RgII strain, a curromycin producing revertant A-4 was obtained, indicating that the structural genes for the curromycin biosynthesis and resistance are retained in the RgII genome. Based on the existence of A-4 and the deletion of the DNA region corresponding to the cloned DNA sequence in the RgII genome, we conclude that the cloned DNA sequence carries a regulatory gene governing curromycin-resistance but not the resistance gene itself. The smallest DNA region in pSHR2 conferring curromycin-resistance was sequenced, and it was found that there were two small open reading frames (ORF) on each strand of the cloned DNA. In-frame fusion of ORFs to the reporter gene *lacZ* revealed that one ORF designated *cre* was indeed translated *in vivo*. The putative gene product deduced from the *cre* ORF is a basic and hydrophilic protein having a calculated molecular weight of 6 kdaltons.

Streptomycetes are Gram-positive and spore-forming bacteria producing various sorts of antibiotics and secondary metabolites. One of the characteristics of the organisms belonging to this genus is the genetic instability found in the chromosome. For example, protoplast regeneration of streptomycetes often leads to changes of phenotypes, and in many cases such mutations are due to the deletion of unstable genes[1,2].

Streptomyces hygroscopicus 358AV2 produces two structurally unrelated antibiotics, carriomycin[3] and curromycin[4,5]. In ordinary culture conditions, the strain produces only carriomycin, but in a particular medium it produces curromycin in addition to carriomycin. Protoplast regeneration of *S. hygroscopicus* 358AV2 gave rise to a pleiotropic mutant RgII, a strain producing neither carriomycin nor curromycin and showing no curromycin-resistance as previously described[6].

We are interested in understanding the relation between the genetic instability, regulation of the curromycin production and its self-resistance. The fact that protoplast regenerant, RgII, is sensitive to curromycin but resistant to carriomycin gave us an opportunity to study the regulation of the curromycin self-resistance mechanism in *S. hygroscopicus* 358AV2. Antibiotic producers may regulate their antibiotic-resistance gene(s) in concert with antibiotic biosynthetic genes[7,8]. For the strains producing the antibiotics such as methylenomycin[7], streptomycin[9], actinorhodin[8] and bialaphos[10], the antibiotic-resistance genes are known to be located close to or within the biosynthetic genes.

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We describe in this paper the molecular cloning and characterization of the gene termed *cre* conferring curromycin-resistance on RgII. It was found by Southern blot analysis that the cloned gene in a plasmid, pSHR2, was deleted from the RgII genome. A DNA fragment cloned in pSHR21 which was one of the curromycin-resistance plasmids derived from pSHR2, contained two small open reading frames (ORF) on each strand of DNA. One ORF was shown to be translated in RgII as revealed by the in-frame fusion of each ORF with the *lac*Z gene of *Escherichia coli*.

Materials and Methods

Strains and Media

The mutant strain, RgII was derived from *S. hygroscopicus* 358AV2 as a protoplast regenerant[5]. *Streptomyces lividans* TK21 was a gift from D. A. HOPWOOD. *Streptomyces* strains were grown in the GPY liquid culture medium[11] at 30°C. R2YE agar plates[12] were used for protoplast regeneration of transformants. MM agar plates[12] were used for the selection of curromycin resistant-transformants.

For construction of the deletion plasmids for dideoxy sequencing and of *lac*Z fusion plasmids, *E. coli* JA221 *hsd*R$^-$ *hsd*M$^+$ *Δtrp*E5 *leu*B6 *lac*Y[13] was used as the host.

Plasmids

The cloning vector pIJ702 carrying the thiostrepton-resistance and tyrosinase genes[12] was kindly provided by D. A. HOPWOOD. pHSG299[14] and pUC19[15] were purchased from Takara Shuzo Co., Ltd. The plasmid pSK10Δ6 carries the *E. coli lac*Z gene lacking the first eight codons of the β-galactosidase gene[16].

Protoplast Regeneration and Transformation

Formation and regeneration of protoplasts and transformation of *S. lividans* TK21 and *S. hygroscopicus* RgII were done according to the laboratory manual of streptomycetes genetics[12].

DNA Isolation

Large scale preparation of pIJ702 and its derivatives was performed as described by HOPWOOD *et al.*[12]. Total DNA of *S. hygroscopicus* was isolated by the method of FISHMAN and HERSHBERGER[17].

Shotgun Cloning of the Genes Conferring Curromycin-resistance

Total DNA of *S. hygroscopicus* 358AV2 was partially digested with *Sau*3 AI and size fractionated by sucrose density gradient centrifugation. Ten μg of 5~10 kb DNA fragments were ligated to 2μg of the *Bgl* II-digested and dephosphorylated pIJ702. The ligation mixture was used for protoplast transformation of RgII. The spore suspensions of the regenerated transformants were plated on MM agar plates containing 50 μg/ml each of curromycin and thiostrepton. Resistance levels of the RgII cells harboring pSHR2 and its derivatives were determined in liquid GPY media.

Southern Blot Analysis

The total DNA of *S. hygroscopicus* digested with *Bcl* I was electrophoresed in an agarose gel and transferred to nitrocellulose filters (Bio-rad). Hybridization probes were made by the nick translation kit (Takara Shuzo Co., Ltd.) and α-^{32}P labeled dCTP (Amersham Japan). Hybridizations were carried out as described by HOPWOOD *et al.*[12], except that formamide was omitted, in 6×SSC containing 5×DENHARDT's solution and 1% SDS at 65°C overnight and the filters were washed four times in 2×SSC at 65°C for 20 minutes at each step.

Determination of Nucleotide Sequence

A curromycin-resistance plasmid pSHR21 derived from pSHR2 was cloned in both pHSG299 (Takara Shuzo Co., Ltd.), a pUC type kanamycin-resistance vector, and pUC19. The DNA regions to be sequenced were deleted to various lengths by the kilo sequence kit (Takara Shuzo Co., Ltd.)[16]. The nucleotide sequence determination was performed by the dideoxy chain termination method[18] using the deaza sequence kit (Takara Shuzo Co., Ltd.).

Construction of In-frame Fusions between *lac*Z and ORF1 or ORF2

Shuttle vectors for *E. coli* and *Streptomyces* containing ORF1 and ORF2 fused to *lac*Z were constructed as follows. A deletion plasmid used for sequencing, pHSG93 (Fig. 4A), was cleaved with *Dra* II and thereafter treated with Klenow DNA polymerase I. After *Hin*d III digestion, the 341bp *Hin*d III-*Dra* II fragment containing the first 28 codons of ORF1 was ligated to pUC19 that had been cleaved with *Acc* I, treated with Klenow DNA polymerase I and subsequently digested with *Hin*d III. The constructed plasmid, pUC19D4, was digested with *Hin*d III and treated with Klenow DNA polymerase I, followed by *Bam*H I digestion to prepare a 354bp *Hin*d III-*Bam*H I fragment. This fragment was cloned in pSK10Δ6[16] between the *Bam*H I and *Sma* I sites to construct pSK10Δ6D4, resulting in an in-frame fusion between ORF1 and *lac*Z. pSK10Δ6D4 digested with *Sph* I and dephosphorylated with calf intestine alkaline phosphatase (CIAP) was ligated to pIJ702 cut with *Sph* I to generate pSHR21ΔD41 and 42.

Plasmids pSHR21ΔS31 and 32 were prepared as follows. Plasmid pHSG92 was cleaved with *Eco*R I and *Sma* I, and the resulting 341bp fragment containing the first 49 codons of ORF2 was cloned between the *Eco*R I and *Sma* I sites of pSK10Δ6. This plasmid pSK10Δ6S3 was cleaved with *Xma* I, treated with Klenow DNA polymerase I, and self-ligated to fuse ORF2 and *lac*Z in-frame. The plasmid thus obtained, pSK10Δ6S31, was cut with *Eco*R I, treated with CIAP, and was ligated to PIJ702 which had been cut with *Sph* I and blunt-ended with T4 DNA polymerase I, followed by addition of *Eco*R I linker.

β-Galactosidase Assay

Streptomyces cells carrying the fusion plasmids, pSHR21ΔD41, 42, pSHR21ΔS31 and 32, were suspended in 10 ml of the GPY medium. The growth of the cells was monitered by measuring the optical density at 600 nm of the 10-fold diluted culture suspension. β-Galactosidase activities were assayed by the method of NAGAMI and TANAKA[19] and expressed as Miller units[20].

Chemicals and Enzymes

Restriction endonucleases, T4 DNA ligase, alkaline phosphatase, the large fragment of DNA polymerase I and T4 DNA polymerase I were purchased from Takara Shuzo Co., Ltd., Toyobo Co., Ltd. or New England Biolabs. Each enzyme was used according to the manufacturer's recommendation. Thiostrepton was kindly supplied by Asahi Chemical Industry. Curromycin was purified as previously described[4]. Curromycin used in this work was a mixture of curromycins A and B.

Results

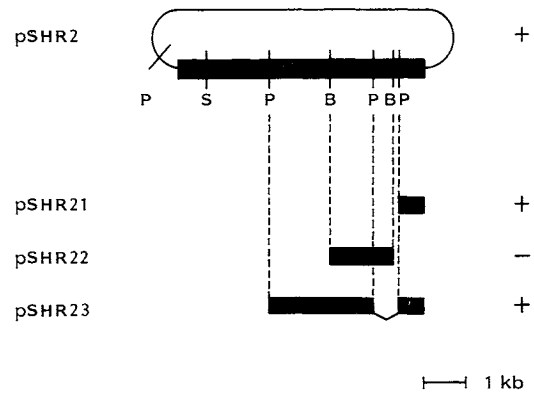
Shotgun Cloning from *S. hygroscopicus* 358AV2 of Genes Conferring Curromycin-resistance on RgII

The curromycin non-producing protoplast regenerant RgII is sensitive to a low concentration of curromycin (1μg/ml). Thus, RgII seemed an appropriate host for cloning genes conferring curromycin-resistance from *S. hygroscopicus* 358AV2. Total DNA of the 358AV2 strain was partially digested with *Sau*3 A1, size fractionated (5$\sim$10 kb), ligated to pIJ702, and introduced into the RgII strain as described in Materials and Methods. Six curromycin-resistant transformants were obtained from about 18,000 transormants. It was found that they contained plasmids which conferred both curromycin- and thiostrepton-resistance upon retransformation into RgII. Restriction analysis of the six plasmids obtained from the transformants revealed that the inserted DNAs had no regions overlapping each other. The plasmids obtained, pSHR1, 2, 3, 4, 5 and 6, carried 6.2, 5.8, 5.2, 2.2, 0.4 and 3.4 kb DNA fragments, respectively, and after subcloning of the resistance gene with *Pst* I, a plasmid, pSHR21, derived from pSHR2 was picked and used for further study since it contained a small fragment (about 500 bp) carrying several restriction sites. The restriction maps of pSHR2 and its derivatives are shown in Fig. 1.

The pSHR2 plasmid and its derivatives conferred relatively low levels of curromycin-resistance

in comparison to the resistance level of the parental strain 358AV2. The Rgll cells harboring pSHR2 were resistant to 40 μg/ml of curromycin, whereas the growth of strain 358AV2 was not affected by a concentration of 100 μg/ml of curromycin. Rgll carrying pSHR2 did not produce detectable levels of curromycin or carriomycin.

Fig. 1. Restriction map of pSHR2 and its derivatives.



The solid bar and the single line indicate the cloned region of the 358AV2 chromosomal DNA and pIJ702, respectively. Filled boxes represent subcloned regions of the 358AV2 chromosomal DNA. "+" and "−" denote curromycin-resistance and sensitivity, respectively. Plasmids pSHR21 and pSHR23 were constructed by *Pst* I digestion and ligation of pSHR2. Plasmid pSHR22 was constructed by insertion of the 1.2 kb *Bam*H I fragment of pSHR2 in the *Bgl* II site of pIJ702. Abbereviations: B, *Bam*H I; P, *Pst* I; S, *Sac* I.

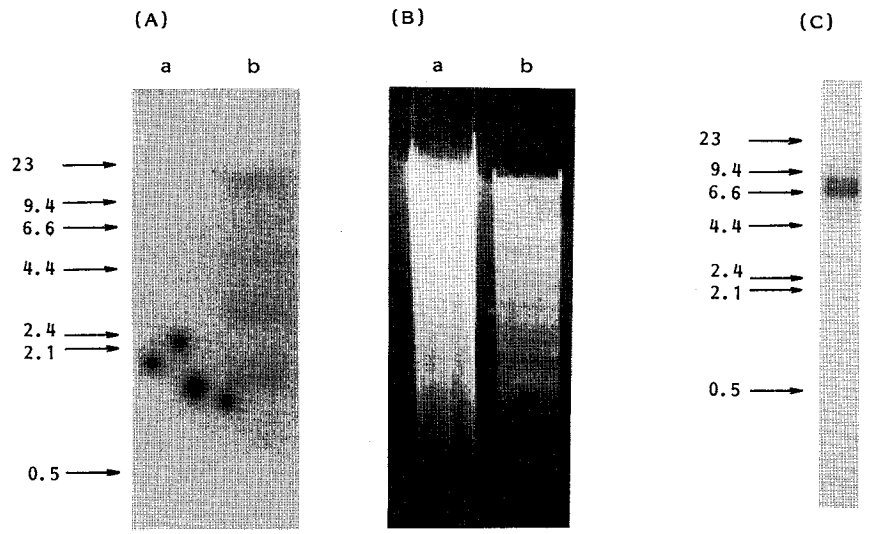
Southern Blot Analysis of 358AV2 and Rgll Genomic DNA using pSHR2

It is known that, in several *Streptomyces* sp., protoplast regeneration often leads to deletion[2] or amplification[21] of specific DNA sequences in the chromosome containing antibiotic-resistance gene(s). To examine whether a gene(s) relating to curromycin-resistance in the Rgll genomic DNA had undergone rearrangement through protoplast regeneration, Southern blot analysis was performed using the whole pSHR2 plasmid as probe because pIJ702 DNA did not hybridize to 358AV2 genomic DNA (data not shown). Total DNA from 358AV2 and Rgll were completely digested with *Bcl* I (the amount of the enzyme used was more than 10 times the concentration which completely digested the chromosomal DNA as judged by the banding pattern), and subjected to hybridization with ^{32}P-pSHR2. Plasmid pSHR2 hybridized to five regions in the genomic DNA from 358AV2 (Fig. 2A), even though we expected that the probe would hybridize to only two DNA fragments since the insert of pSHR2 has one *Bcl* I site. The pSHR2 probe did not hybridize to the Rgll genomic DNA, indicating that the DNA cloned in pSHR2 was deleted from the Rgll genome. Taking account of the correlation between the loss of curromycin-resistance and the deletion in Rgll of the DNA region cloned in pSHR2, we suspected that the DNA deletion might be responsible for the sensitivity of Rgll to curromycin. Furthermore pSHR21 hybridized to two bands in the total DNA from 358AV2 digested by *Bam*H I. Since DNA inserted in pSHR21 had no *Bam*H I site, the result shows that this DNA region has related sequence in 358AV2 genomic DNA (Fig. 2C).

Plasmid pSHR2 Encodes Regulatory Genes for Curromycin-resistance

A curromycin producing and resistant revertant, A-4, was obtained when the curromycin non-producing and sensitive mutant, Rgll, was treated with ethidium bromide. The A-4 strain produced both curromycin (45 μg/ml) and carriomycin (0.6 μg/ml) but this phenotype was unstable, *i.e.*, the productivity of the antibiotics was gradually lost[6]. These results show that the structural gene(s) for self-resistance to curromycin is retained in the chromosome of Rgll in a cryptic form, and that the expression of the gene(s) was induced by ethidium bromide, although the induction mechanism is currently unknown. The finding that the DNA sequence cloned in pSHR2, which can confer curromycin-resistance on Rgll, was deleted from the Rgll genome suggested that pSHR2 contained a genetic element inducing self-resistance. Attempts to confer curromycin-resistance on *S. lividans* TK21 with pSHR2 were unsuccessful, suggesting

Fig. 2. Southern blot analysis of the *Streptomyces hygroscopicus* 358AV2 and RgII genomic DNA using whole pSHR2 and pSHR21 as a probe.



The patterns of hybridization and agarose gel electrophoresis are shown in (A) and (B), respectively. (C) shows the hybridization patterns of pSHR21 to 358AV2 DNA digested with *Bam*H I. The genomic DNA of each strain was digested with *Bcl* I and *Bam*H I completely and transferred to a nitrocellulose membrane filter. The filter was washed in 2× SSC at 65°C. Lanes: a, RgII; b, 358AV2. Sizes of λ DNA digested with *Hin*d III are shown on the left.

Fig. 3. Nucleotide sequence of the DNA fragment inserted in pSHR21.

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                    Pst I                                   Sma I
                    CTGCAGACGGCGCAGCTCGCTCTCGTACCGGACCCGGGGCAGTCGGCC    48
                    GACGTCTGCCGCGTCGAGCGAGAGCATGGCCTGGGCCCCGTCAGCCGG

CAAAGCCGCGGTCCCCATGTGGCCAGTGTCGCACCGGGCGGACCGCTCCGCCGCCTCCCCCGGCGG    114
GTTTCGGCGCCAGGGGTACACCGGTCACAGCGTGGCCCGCCTGGCGAGGCGGCGGAGGGGGCCGCC

            Sma I
TCACCGCCGCGGCCCCGGGCGAGACCTGCCTGCTGACCCTTCCACCCGCACACGCTGAAGGCATAA    180
AGTGGCGGCGCCGGGGCCCGCTCTGGACGGACGACTGGGAAGGTGGGCGTGTGCGACTTCCGTATT
***ArgArgProGlyProArgSerArgGlyAlaSerGlyGluValArgValArgGlnLeuCysLeu

AGCGCCGAGGGCTGCCCGCTTCTCGTGGGCCCGGATTGCACGGTGCGCGACTTCACGCCGCTTCTC    246
TCGVGGCTCCCGACGGGCGAAGAGCACCCGGGCCTAACGTGCCACGCGCTGAAGTGCGGCGAAGAG
AlaGlyLeuAlaAlaArgLysGluHisAlaArgIleAlaArgHisAlaValGluArgArgLysGlu

(ORF1)
 MetSerValValAlaLeuGlyAlaThrSerIleThrProProHisGlyProGluSerGlnGlyAr
CGTGTCTGTTGTCGCCCTCGGTGCCACTTCCATAACACCACCACACGGCCCGGAGAGCCAGGGCCG    312
GCACAGACAACAGCGGGAGCCACGGTGAAGGTATTGTGGTGGTGTGCCGGGCCTCTCGGTCCCGGC
ThrAspThrThrAlaArgProAlaValGluMet
                          (ORF2)

            Dra II
gProPheProAlaArgGlyProValArgProSerAlaArgAlaArgProValProLeuTrpThrTy
ACCGTTCCCTGCCCGAGGGCCTGTCCGACCATCCGCGCGAGCCCGGCCGGTGCCACTGTGGACATA    378
TGGCAAGGGACGGGCTCCCGGACAGGCTGGTAGGCGCGCTCGGGCCGGCCACGGTGACACCTGTAT

rGlyTyrSerGluGlyThrSerArgAlaGlyArgArgTrpGly***
CGGGTACTCAGAGGGGACGAGCCGCGCAGGAAGGCGGTGGGGATGATGTCGGTCTCGCTGGTCGTG    444
GCCCATGAGTCTCCCCTGCTCGGCGCGTCCTTCCGCCACCCCTACTACAGCCAGAGCGACCAGCAC

GGGGTCGACGGATC                                                      458
CCCCAGCTGCCTAG
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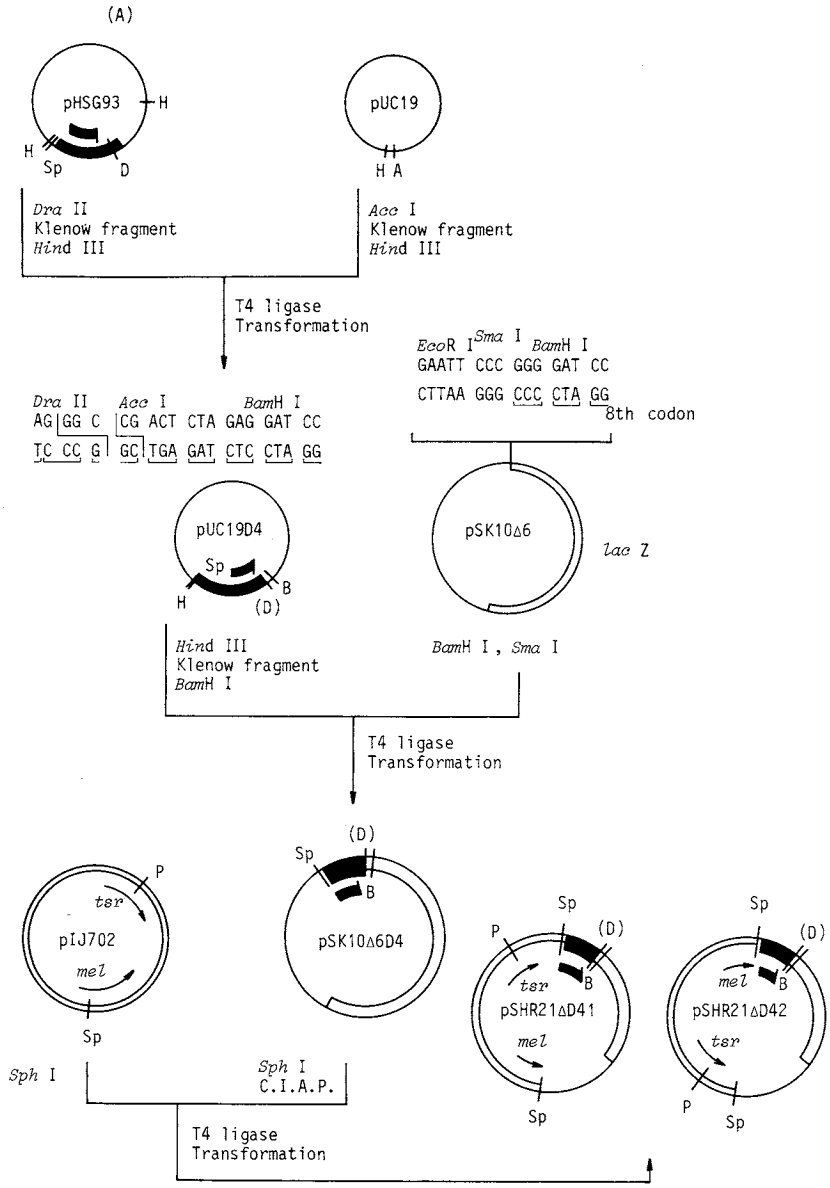
The deduced amino acid sequences of ORF1 and ORF2 are shown above and below the nucleotide sequences, respectively.

Fig. 4. Construction strategy of the fusion (A) ORF1, (B) ORF2.

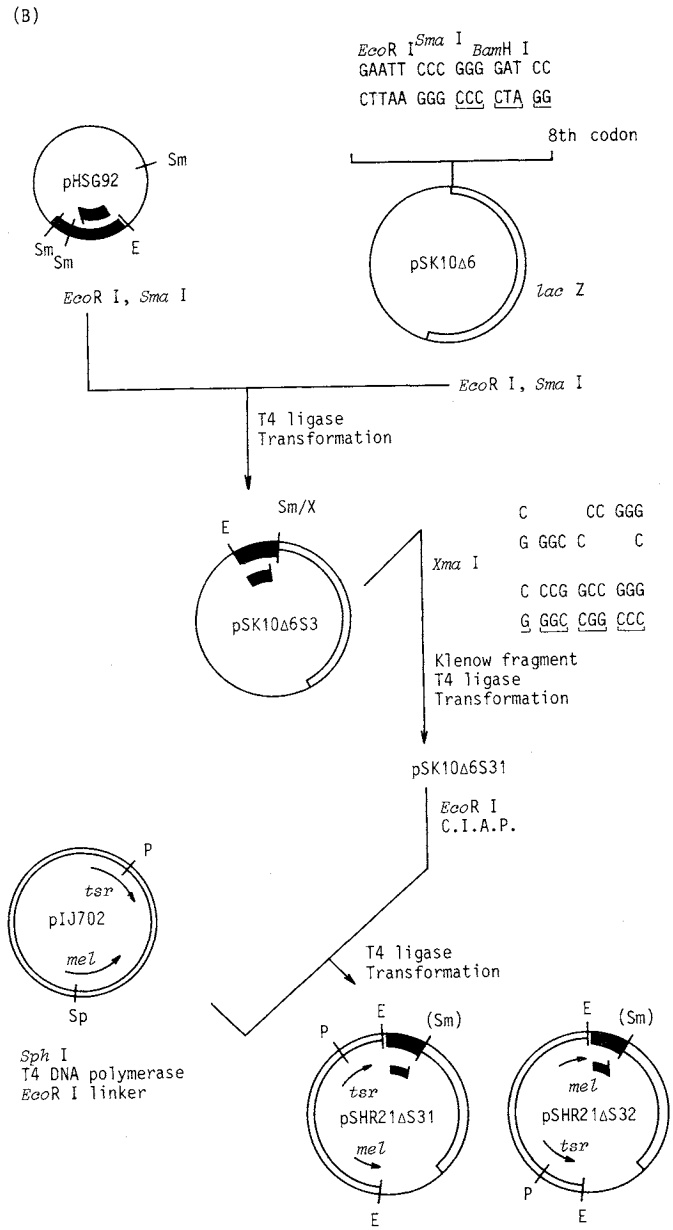


that the cloned sequence did not encode curromycin-resistance itself, but had a regulatory function governing curromycin-resistance.

Determination of the Nucleotide Sequence Conferring Resistance to Curromycin

Plasmid pSHR21 has the shortest DNA fragment among the subcloned DNA fragments which confer curromycin-resistance on Rgll. pSHR21 carried a DNA fragment of about 500 bp, whose nucleotide sequence was determined. From a computer analysis of the sequence, one ORF on each strand was found, ORF1 and ORF2 (Fig. 3). ORF1 consists of 58 codons starting from GTG (Met) and ending in GGA

plasmids between the *lacZ* and ORF1 or ORF2.



Details of construction are shown in Materials and Methods. The arrows associated with *tsr* and *mel* indicate the direction of transcription. The thick arrows show the direction of ORF1 and ORF2. Abbreviations: A, *Acc* I; B, *Bam*H I; D, *Dra* II; E, *Eco*R I; H, *Hind* III; P, *Pvu* II; Sm, *Sma* I; Sp, *Sph* I; X, *Xma* I; *tsr*, thiostrepton-resistance gene; *mel*, tyrosinase gene.

(Gly), whereas ORF2 consisted of 54 codons, starting from ATG (Met) and ending in CGG (Arg). *Streptomyces* species contain a high GC content of DNA, and the GC content of the third positions of codons is around 90%[22]. In contrast, ORF1 and ORF2 were found to contain GC contents of only 64% and 69% at the third positions of the codons, respectively.

Table 1. Expression of β-galactosidase activity in RgII harboring the *lac*Z gene in-frame fused with ORF1 and ORF2.

	pIJ702	pSHR21ΔD41	pSHR21ΔD42	pSHR21ΔS31	pSHR21ΔS32
1 day					
Growth (OD_{600})[a]	0.27	0.36	0.25	0.31	0.29
Miller units	0.22	1.20	1.31	0.18	0.25
2 days					
Growth (OD_{600})[a]	0.48	0.37	0.73	0.51	0.40
Miller units	0.15	0.99	1.98	0.21	0.16

β-Galactosidase activities were determined as described in Materials and Methods and are shown as Miller units.
[a] The growth of cells was monitored by OD_{600} after the culture suspensions were diluted 10-fold.

Expression of β-Galactosidase by the Fusion of the *lac*Z Gene with ORF1 and ORF2

To determine whether ORF1 and ORF2 are indeed expressed *in vivo*, we constructed in-frame fusion plasmids between either ORF1 or ORF2 and the *E. coli lac*Z gene lacking the first eight codons as shown in Fig. 4. Strategies of the fusion construction are shown in Figs. 4 (A) and (B).

The plasmids containing fusions between ORF1 or ORF2 and the *lac*Z gene of *E. coli* in both directions with respect to pIJ702 are designated pSHR21ΔD41 and 42 or pSHR21ΔS31 and 32, respectively. These plasmids were introduced into RgII, and β-galactosidase activities were measured. The results are shown in Table 1. RgII carrying pSHR21ΔD41 and 42 showed higher levels of β-galactosidase activity, and attained a 6- and 13-fold enhanced level of the activity in 2 days, respectively. In contrast, RgII carrying pSHR21ΔS31 and 32 showed only control levels of β-galactosidase activity. These results show that the ORF protein as deduced from the DNA sequence does exist *in vivo*. Accordingly, we conclude that the ORF1 is responsible for curromycin-resistance in RgII transformants. The gene encoding ORF1 is named curromycin-resistance enhancement (*cre*).

Discussion

We cloned in a curromycin-sensitive and non-producing variant strain *S. hygroscopicus* RgII a DNA sequence conferring resistance to curromycin up to the concentration of 40 μg/ml in the liquid GPY medium. The DNA fragment in the constructed plasmid, pSHR2, was further shortened by subcloning experiments to produce pSHR21. The nucleotide sequence analysis showed the presence on pSHR21 of an ORF encoding a small protein which was shown to be expressed in the curromycin-resistant RgII transformant. Southern blot analysis revealed that the DNA sequence inserted in pSHR2 was deleted from the RgII genome. Transformation of pSHR2 into *S. lividans* TK21 which does not produce curromycin could not confer curromycin-resistance on this strain. These results show that the small ORF carried on the plasmid pSHR2 is involved in the curromycin-resistance.

Plasmid pSHR2 hybridized to five DNA fragments, although it was expected to hybridize to two DNA fragments, suggesting the possibility that the gene conferring curromycin-resistance belongs to a gene family in which individual genes share homology with each other. In fact, pSHR21 hybridized to two DNA fragments, although pSHR21 was expected to hybridize to single fragment. Furthermore, these results suggest that the DNA region flanking the gene conferring curromycin-resistance has homologies with serveral DNA regions in the parental strain, 358AV2. It is of interest to note that this DNA region was found to be deleted in the RgII genome. One possible explanation for this is that an extrachromosomal element(s) is involved in curromycin-resistance, and has been lost in the RgII cells. Recently a plasmid, SCP1, which codes the methylenomycin biosynthetic enzymes was detected by the OFAGE method as a

giant linear molecule[23]. Similar attempts to detect a plasmid in the 358AV2 strain including the OFAGE method and the conventional procedures such as the cleared lysate and alkaline lysis methods failed to reveal such DNA elements (data not shown).

Determination of the nucleotide sequence of the cloned DNA in pSHR21 revealed that the *cre* gene lies on an ORF encoding 58 amino acids. The codon usage pattern on the *cre* is exceptional taking that of the *Streptomyces* genes into consideration. The *cre* ORF appears to lack an SD sequence. This is not unprecedented, however, since several antibiotic-resistance genes such as *aph* of *Streptomyces fradiae*[22], *ermE* of *Streptomyces erythraeus*[24] and *sta* of *Streptomyces lavendulae*[25] are devoid of an SD sequence.

The in-frame fusion of *cre* with the *E. coli lacZ* gene strongly suggests that it was translated *in vivo*. To verify that the *cre* protein indeed exists *in vivo*, it will be necessary to purify this protein or the fusion protein between the *cre* and the *lacZ* genes and to determine the *N*-terminal amino acid sequence. The deduced protein from *cre* has a calculated molecular weight of 6,189. It is a highly charged (18.9% of the amino acids are charged) and basic (15.5% of the amino acids are basic) protein. The average hydropathy for the amino acids encoded by *cre* was -0.59 as calculated by the method of KYTE and DOOLITTLE[26]. The finding that a small protein such as *cre* is involved in the regulation of gene expression is not unusual. For example it has been reported that small proteins are involved in regulation of extracellular proteases and levansucrase in *Bacillus subtilis*. These include the *prt*R[19], *sac*Q[27] and *sen*N[28] genes coding for proteins consisting of 60, 46 and 60 amino acids, respectively. Overexpression of the *prt*R and *sac*Q gene products causes overproduction of extracellular proteases and levansucrase. The gene products of *prt*R, *sac*Q and *sen*N are also hydrophilic and contain the high percentage of charged amino acids like the putative *cre* gene product. Both the *prt*R and *sac*Q gene products were shown to activate transcription of the target genes[29,30]. However, neither *sac*Q nor *prt*R is essential for the production of the wild type level of the enzymes as shown by gene disruption experiments[27,31]. As described in the results, we obtained five other plasmids conferring curromycin-resistance. The cloned DNA fragments were further shortened to less than 1 kb by subcloning experiments (data not shown), suggesting that they also encode small proteins. From these findings and by analogy with the regulation of the extracellular protease production in *B. subtilis*, we infer that the curromycin-resistance is regulated by multiple genes and that the indivudual genes, when present on a multicopy plasmid, render the non-curromycin-producing cells of strain Rgll resistant to the antibiotic. The mechanism of how the *cre* gene product regulates curromycin-resistance in Rgll will be the subject of future work.

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