Cloning and sequence analysis of a plasmid-encoded chloramphenicol acetyltransferase gene from *Staphylococcus intermedius*

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The chloramphenicol acetyltransferase gene (*cat*) of a 3.9 kb chloramphenicol resistance (Cm^R) plasmid from *Staphylococcus intermedius*, designated pSCS1, was cloned into an *Escherichia coli* plasmid vector. Sequence analysis revealed a high degree of base similarity with the *cat* gene of the *S. aureus* Cm^R plasmid pC221 but there were several differences in the regulatory region. A lesser degree of similarity was observed between the *cat* gene of the *S. intermedius* plasmid and the *cat* gene of the *S. aureus* plasmid pC194.

Introduction

Chloramphenicol (Cm) was introduced into clinical use as a broad spectrum antibiotic in the 1950s. Because of its toxicity in bone marrow (Yunis, 1973), its use in man was limited to the control of life-threatening infections (Shaw, 1984). However, in veterinary practice Cm is used to control many animal infections. Bacterial resistance to Cm was detected soon after its introduction into clinical use, as seen with other antibiotics. In staphylococci, the determinants for chloramphenicol resistance (Cm^R) occur exclusively on small plasmids (Lyon & Skurray, 1987). The Cm^R plasmids from human *Staphylococcus aureus* strains have been studied intensively, and three distinct plasmid families have been differentiated (Gillespie & Skurray, 1988).

However, little is known about Cm^R in staphylococcal species pathogenic to animals. Recently, Cm^R plasmids have been isolated from 'equine' *S. sciuri* (Schwarz *et al.*, 1990a), 'canine' *S. epidermidis* (Schwarz *et al.*, 1990b), 'porcine' *S. hyicus* (Schwarz *et al.*, 1989a) and 'canine' *S. intermedius* strains (Schwarz *et al.*, 1989b). All staphylococcal Cm^R plasmids encode a common resistance mechanism, namely an inducible Cm acetyltransferase (CAT).

This paper reports the sequence of the *cat* gene and regulatory region carried by a 3.9 kb *S. intermedius*

plasmid. The relationship of this *cat* gene to those of the prototype Cm^R plasmids pC221 and pC194 from *S. aureus* is also discussed.

Methods

Bacteria and growth conditions. A *S. intermedius* strain was isolated from a purulent skin infection of a dog and identified with the ATB 32 Staph-system (Bio Merieux). *S. intermedius* cultures were prepared by overnight incubation at 37 °C on blood agar base (Gibco) supplemented with 7.5% (w/v) sheep blood. For plasmid isolation the bacteria were grown in brain heart infusion broth (Merck) overnight at 37 °C on a rotary shaker at 175 r.p.m. Cm^R of this *S. intermedius* strain was determined as previously described (Schwarz *et al.*, 1989b).

Plasmid DNA isolation. A previously described modification of an alkaline lysis procedure was used (Schwarz & Blobel, 1989) for small-scale plasmid preparation of the *S. intermedius* strain and the *S. aureus* pSCS1 transformant. For large scale plasmid preparations the Qiagen system (Diagen) was modified. The modification involved disruption of the staphylococcal cell walls by incubation in P1-buffer supplemented with 40 µg ml⁻¹ lysostaphin (Sigma) for 45 min at 37 °C.

Plasmid transformation. The *S. intermedius* plasmid was transferred into *S. aureus* RN4220 (Novick, 1967) by protoplast transformation (Schwarz *et al.*, 1989a; Chang & Cohen, 1979). Transformants were selected by incubation on DM3 regeneration plates (Chang & Cohen, 1979), supplemented with 15 µg Cm ml⁻¹, for 48–72 h at 37 °C.

Cloning experiments and sequence analyses. Restriction endonucleases were obtained from Boehringer and used according to the manufacturer's instructions. Standard DNA manipulation techniques were employed (Sambrook *et al.*, 1989). pSCS1 DNA was cloned in pBluescript II SK⁺ (Stratagene) using *Escherichia coli* XL-1 blue (Stratagene) as a host.

A 1.55 kb *Mbo*I fragment of pSCS1 was cloned in both orientations into the single *Bam*HI site of the plasmid vector and a 0.9 kb *Taq*I–*Mbo*I fragment was used for sequence analysis. DNA sequencing was

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performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) modified for double-stranded templates (Sambrook *et al.*, 1989) using T_7 DNA polymerase (Tabor & Richardson, 1987) and [α - 35 S]dATP [> 600 Ci mmol $^{-1}$ (22 TBq mmol $^{-1}$)] from Amersham. DNA was sequenced on both strands using overlapping oligonucleotide primers. A first set of primers was purchased from Stratagene. From the sequence data obtained with these primers, four synthetic 17mer oligonucleotide primers were designed, synthesized with an Applied Biosystems 380 B DNA synthesizer and used for sequence analyses (Brenner & Shaw, 1985).

Results and Discussion

A Cm-resistant strain of *S. intermedius* was isolated from an infected dog. It harboured a single plasmid of 3.9 kb which carried the genetic determinant for Cm^R as demonstrated by transformation into *S. aureus*. A restriction map of this plasmid, designated pSCS1, was constructed (Fig. 1) which allowed comparisons between pSCS1 and the three prototype Cm^R plasmids pC221, pC223 and pC194 from *S. aureus*. Similarities between pC221 and pSCS1 were found in a 2 kb region which encodes, in pC221, CAT and a protein required for plasmid replication (REP). Subcloning showed that the *cat* gene was located within a 1.55 kb *Mbo*I fragment. This fragment was cloned in both orientations into pBluescript II SK⁺. Subsequently, *E. coli* XL-1 blue cells transformed with these recombinant plasmids were tested for Cm^R. In one orientation, *E. coli* XL-1 blue demonstrated Cm^R at 15 μ g Cm ml $^{-1}$ while in the other orientation a higher level of Cm^R occurred (80 μ g Cm ml $^{-1}$).

As predicted from the restriction maps, DNA sequence analysis confirmed a high degree of similarity between the *cat* genes of pSCS1 and pC221. A 901 bp *Taq*I-*Mbo*I fragment contained the complete *cat* structural gene and the regulatory region located about 80 bp 5' to the coding sequences. This region exhibited 97.9% identity with the corresponding region of pC221. Of the 19 variant bases, eight were within the *cat* gene coding region. Only five caused alterations in the amino acid sequence: Leu, Asn and Met in the pC221 CAT variant were replaced by the functionally similar residues Phe, Ser and Ile in CAT from pSCS1 (changes at positions 565, 689 and 810, respectively). The changes at positions 832 and 834 led to the replacement of His by Lys. Substitutions at positions 441, 759 and 762 did not cause changes in the amino acid sequences. The active centre of CAT, His 196 (Leslie *et al.*, 1988), was not affected. The other 11 alterations in the nucleotide sequence of pSCS1 were in non-coding regions (Fig. 2).

However, five base changes occurred in the regulatory region which comprises two Shine-Dalgarno (SD) sequences (SD1, positions 129–136 and SD2, positions 193–198), an open reading frame for a small peptide of

Fig. 1. Circular restriction map of the Cm^R plasmid pSCS1 from *S. intermedius*. The black box indicates the region which showed extensive homologies to the REP/CAT region of the prototype plasmid pC221 from *S. aureus*.

nine amino acids (144–173) and inverted repeats at positions 163–175 and 189–201. In mRNA the inverted repeats form a stem-loop structure (Brückner & Matzura, 1985; Lovett, 1990) located immediately upstream from the CAT coding region (Fig. 3). This relatively, stable stem-loop is a transcription terminator and SD2 within the stem is not accessible to ribosomes (Lovett, 1990). In pC221, the relative stability of the stem-loop was $\Delta G = -18.0$ kcal mol $^{-1}$ (-75.3 kJ mol $^{-1}$; Tinoco *et al.*, 1973). The base change from A in pC221 to T in pSCS1 at position 201 would lengthen the inverted repeat by 1 bp and would increase the relative stability of the stem-loop to -19.8 kcal mol $^{-1}$ (-82.8 kJ mol $^{-1}$).

Moreover, two other important base substitutions occurred within SD1. This region of the mRNA interacts with its complement in the 16S rRNA. This interaction is essential for the subsequent translation of the *cat* gene. Despite the stem-loop structure, ribosomes are presumably able to bind to SD1. Translation of the small open reading frame, which is highly conserved in all sequenced *cat* genes in the pC221 family (Brenner & Shaw, 1985; Brückner & Matzura, 1985), might be expected to destabilize the stem-loop structure (Brückner & Matzura, 1985; Lovett, 1990), making SD2 accessible to ribosomes and allowing translation of the *cat* structural gene (Lovett, 1990). The exchanges from G in pC221 to A in pSCS1 at position 130, and from C in pC221 to T in pSCS1 at position 136, within SD1, would increase the free energy of the binding of mRNA to 16S rRNA from -11.4 kcal mol $^{-1}$ (-47.7 kJ mol $^{-1}$) in the case of pC221, to -17.8 kcal mol $^{-1}$ (-74.5 kJ mol $^{-1}$) in the case of

10 20 30 40 50 60
TaqI
 TCGACTTTAAAGCGAACGAAAAACAATTGCAAAACAGATTGATTTTGGCAACGTGAA
 70 80 90 100 110 120
 TTTAGGTTTTTGAAGTAAATAAGTTTTATTTTATAAAAAATTGCTAATTCAGTATAATTA
 130 140 150 160 170 180
 ATATTTACGAGGTGATATAACGTATGAAAAATCAGAGGATTATTCCTCCTAAATACAAA
 SD1 M K K S E D Y S S (1)
 190 200 210 220 230 240
 CATTTAAAATTTAGGAGGAATTTATATATGACTTTTAATATTATCAAATTAGAAAATTGG
 SD2 M T F N I I K L E N W (2)
 250 260 270 280 290 300
 GATAGAAAAGAATATTTGAACACTATTTTAACCAGCAAACCTACGTATAGCATTACTAAA
 D R K E Y F E H Y F N Q Q T T Y S I T K
 310 320 330 340 350 360
 GAAATTGATATTACTTTGTTTAAAGATATGATAAAAAAGAAAGGATATGAAATTTATCCT
 E I D I T L F K D M I K K K G Y E I Y P
 370 380 390 400 410 420
 TCTTTGATTATGCAATTATGGAAGTTGTAATAAAAAATAAAGTGTTTAGAACAGGAATT
 S L I Y A I M E V V N K N K V F R T G I
 430 440 450 460 470 480
 AATAGTGAGAATAAATTAGGCTATTGGGATAAGTTAAATCCTTTGTATACAGTTTTTAAT
 N S E N K L G Y W D K L N P L Y T V F N *AclI*
 490 500 510 520 530 540
 AAGCAAACCTGAAAAATTTACTAACATTTGGACTGAATCTGATAACAACTTCACTTCTTTT
 K Q T E K F T N I W T E S D N N F T S F
 550 560 570 580 590 600
 TATAATAATTATAAAATGACTTGTTTGAATATAAAGATAAAGAAGAAATGTTTCCTAAA
 Y N N Y K N D L F E Y K D K E E M F P K
 610 620 630 640 650 660
 AAACCGATACCTGAAAACACCATACCGATTCAATGATTCCTTGGATTGATTTTAGTTCA
 K P I P E N T I P I S M I P W I D F S S
 670 680 690 700 710 720
 TTTAATTTAAACATTGGTAACAATAGCAGCTTTTTATTGCCTATTATTACGATAGGTAAA
 F N L N I G N N S S F L L P I I T I G K
 730 740 750 760 770 780
 TTTTATAGTGAGAATAATAAAATTTATATACCAGTTGCCTTACAGCTTCATCATGCTGTA
 F Y S E N N K I Y I P V A L Q L H H A V
 790 800 810 820 830 840
 TGTGATGGTTACCATGCTTCATTATTTATAAATGAATTTCAAGATATAATTAAGAAGGTA
 C D G Y H A S L F I N E F Q D I I K K V *BstFI*
 850 860 870 880 890 900
 GATGATTGGATTTAGTTTTTAGATTTTGGGAGTGAATTTAATTTTATACACGTAAGTGATC
 D D W I *MboI*

Fig. 2. DNA sequence of the 0.9 kb *TaqI*-*MboI* fragment containing the *cat* structural gene and its regulatory region, presented as the non-coding strand. Two putative Shine-Dalgarno sequences (SD1, SD2) are marked. The arrows indicate the extent of the inverted repeats (1) and (2). Positions of basepair changes with respect to pC221 are marked by vertical lines. The amino acid sequences of the two open reading frames, deduced from the respective nucleotide sequences, are displayed in the single-letter code.

Fig. 3. Probable stem-loop configuration in mRNA transcribed from the *cat* gene control region. The two putative SD sequences (SD1, SD2) as well as the inverted repeats (1) and (2) are marked. The small arrows indicate the positions in which basepair differences occur with respect to pC221. The dotted line indicates the coding sequence for a nine amino acid peptide.

pSCS1. These two mutations probably result in a stronger translation initiation signal. However, the mutation at position 210 would result in a more stable RNA stem-loop, which might represent a stronger block for initiation of translation of the *cat* gene. Thus, the mutations seem to cause opposite effects, with a stronger translation initiation signal being required to destabilize a more stable RNA stem-loop structure. The remaining two basepair changes within the regulatory region, at positions 177 and 181, should not cause any conformational or energetic changes.

Comparisons of the DNA sequence of the *cat* fragment of pSCS1 with the corresponding region of pC194 from *S. aureus* (Horinouchi & Weisblum, 1982) revealed 62.9% identity, while the amino acid sequence of CAT from pSCS1 shared only 53.5% identity with CAT from pC194.

On the basis of the similarities in the restriction map of the REP/CAT region of pC221 and pSCS1, the *S. intermedius* plasmid could be assigned to the pC221 family of staphylococcal Cm^R plasmids. This was confirmed by comparisons of nucleotide sequences. The presence of similar REP/CAT regions in Cm^R plasmids of the pC221 family isolated from different staphylococcal species in different geographical areas had been observed before (Gillespie & Skurray, 1988; Schwarz *et al.*, 1989a, b, 1990a, b). The widespread occurrence of this highly conserved REP/CAT region might be a consequence of plasmid mobilization and subsequent interplasmid recombination. The presence of the *mob* genes and the mobilization site *oriT* in pC221 (Novick, 1989) might have allowed the mobilization of pC221 into other species by conjugative plasmids (Novick, 1989).

Site-specific interplasmid recombination might occur (Novick, 1989) with plasmids resident in the new hosts. Two sequences which flank the REP/CAT region, namely a recombination site B and the CAT terminator, were shown to be involved in such recombinational events and might also be responsible for spreading the REP/CAT region among other plasmids, thus extending the pC221 family of plasmids (Gillespie & Skurray, 1988; Projan *et al.*, 1985; Novick *et al.*, 1984). In this way, new plasmids could be formed (Lampson & Parisi, 1986) which are better adapted to the new bacterial host.

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References

- BRENNER, D. G. & SHAW, W. V. (1985). The use of synthetic oligonucleotides with the universal templates for rapid DNA sequencing: results with staphylococcal replicon pC221. *EMBO Journal* **4**, 561–568.
- BRÜCKNER, R. & MATZURA, H. (1985). Regulation of the inducible chloramphenicol acetyltransferase gene of *Staphylococcus aureus* plasmid pUB112. *EMBO Journal* **4**, 2295–2300.
- CHANG, S. & COHEN, S. N. (1979). High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Molecular and General Genetics* **168**, 111–115.
- GILLESPIE, M. T. & SKURRAY, R. A. (1988). Structural relationships among chloramphenicol-resistance plasmids of *Staphylococcus aureus*. *FEMS Microbiology Letters* **51**, 205–210.
- HORINOUCHI, S. & WEISBLUM, B. (1982). Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. *Journal of Bacteriology* **150**, 815–825.
- LAMPSON, B. C. & PARISI, J. T. (1986). Nucleotide sequence of the constitutive macrolide-lincosamide-streptogramin B resistance plasmid pNE131 from *Staphylococcus epidermidis* and homologies with *Staphylococcus aureus* plasmids pE194 and pSN2. *Journal of Bacteriology* **167**, 888–892.
- LESLIE, A. G. W., MOODY, P. C. E. & SHAW, W. V. (1988). Structure of chloramphenicol acetyltransferase at 1.75 Å resolution. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 4133–4137.
- LOVETT, P. S. (1990). Translational attenuation as the regulator of inducible *cat* genes. *Journal of Bacteriology* **172**, 1–6.
- LYON, B. R. & SKURRAY, R. A. (1987). Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. *Microbiological Reviews* **51**, 88–134.
- NOVICK, R. P. (1967). Properties of a cryptic high frequency transducing phage in *Staphylococcus aureus*. *Virology* **33**, 155–166.
- NOVICK, R. P. (1989). Staphylococcal plasmids and their replication. *Annual Review of Microbiology* **43**, 537–565.
- NOVICK, R. P., PROJAN, S. J., ROSENBLUM, W. & EDELMAN, I. (1984). Staphylococcal plasmid cointegrates are formed by host- and phage-mediated general *rec* systems that act on short regions of homology. *Molecular and General Genetics* **195**, 374–377.
- PROJAN, S. J., KORNBLUM, J., MOGHAZEH, S. L., EDELMAN, I., GENNARO, M. L. & NOVICK, R. P. (1985). Comparative sequence and functional analysis of pT181 and pC221, cognate plasmid replicons from *Staphylococcus aureus*. *Molecular and General Genetics* **199**, 452–464.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

- SANGER, F., NICKLEN, S. & COULSEN, A. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SCHWARZ, S. & BLOBEL, H. (1989). Plasmids and resistance to antimicrobial agents and heavy metals in *Staphylococcus hyicus* from pigs and cattle. *Journal of Veterinary Medicine* **36**, 669–673.
- SCHWARZ, S., CARDOSO, M. & BLOBEL, H. (1989a). Plasmid-mediated chloramphenicol resistance in *Staphylococcus hyicus*. *Journal of General Microbiology* **135**, 3329–3336.
- SCHWARZ, S., CARDOSO, M. & BLOBEL, H. (1989b). Plasmid-encoded resistance to chloramphenicol in 'canine' *Staphylococcus intermedius* isolates. *Medical Science Research* **17**, 451–453.
- SCHWARZ, S., CARDOSO, M. & BLOBEL, H. (1990a). Detection of a novel chloramphenicol resistance plasmid from 'equine' *Staphylococcus sciuri*. *Journal of Veterinary Medicine* **37**, 674–679.
- SCHWARZ, S., CARDOSO, M., GRÖLZ-KRUG, S. & BLOBEL, H. (1990b). Common antibiotic resistance plasmids in *Staphylococcus aureus* and *Staphylococcus epidermidis* from human and canine infections. *Zentralblatt für Bakteriologie* **273**, 369–377.
- SHAW, W. V. (1984). Chloramphenicol acetyltransferase: enzymology and molecular biology. *CRC Critical Reviews of Biochemistry* **14**, 1–46.
- TABOR, S. & RICHARDSON, G. C. (1987). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 4767–4771.
- TINOCO, I. JR, BORER, P. N., DENGLER, B., LEVINE, M. D., UHLENBECK, O. C., CROTHERS, D. M. & GRALLA, J. (1973). Improved estimation of secondary structure in ribonucleic acids. *Nature New Biology* **246**, 40–41.
- YUNIS, A. A. (1973). Chloramphenicol-induced bone marrow suppression. *Seminars in Hematology* **10**, 225.