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Molecular Cloning, Purification, and Properties of a Plasmid-Encoded Chloramphenicol Acetyltransferase from *Staphylococcus haemolyticus*

STEFAN SCHWARZ* AND MARISA CARDOSO

Institut für Bakteriologie und Immunologie der Justus Liebig Universität Giessen, Frankfurterstrasse 107, 6300 Giessen, Federal Republic of Germany

Received 10 January 1991/Accepted 12 April 1991

A small chloramphenicol resistance (Cm^r) plasmid of approximately 3.75 kb, designated pSCS5, was isolated from *Staphylococcus haemolyticus*. This plasmid encoded an inducible chloramphenicol acetyltransferase (CAT; EC 2.3.1.28). The *cat* gene of pSCS5 was cloned into the *Escherichia coli* plasmid vector pBluescript $SKII^+$. It differed in its nucleotide sequence and deduced amino acid sequence from the *cat* genes described previously in staphylococci and other gram-positive bacteria. The CAT enzyme was purified from cell-free lysates by ammonium sulfate precipitation, ion-exchange chromatography, and fast protein liquid chromatography. The native enzyme had an M_r of 70,000 and was composed of three identical subunits, each with an M_r of approximately 23,000. Its isoelectric point was at pH 6.15. CAT from pSCS5 exhibited K_m values of 2.81 and 51.8 μM for chloramphenicol and acetyl coenzyme A, respectively. The optimum pH for activity was 7.8. CAT encoded by pSCS5 proved to be relatively heat stable, but sensitive to mercury ions. The observed differences in the nucleotide sequence and the biochemical characteristics of the enzyme allowed the identification of the pSCS5-encoded CAT from *S. haemolyticus* as a CAT variant different from those described previously in gram-positive bacteria.

Staphylococcus haemolyticus is involved in a variety of human infections, such as septicemia and urinary tract and wound infections (9, 10, 12, 17, 24). Its role in animal infections has not been determined (17). Antibiotics are commonly used to control *S. haemolyticus* infections (10, 12, 17). Several investigations have been conducted to study the antimicrobial resistance patterns of *S. haemolyticus* (9, 10, 24), and they have shown that the organism is resistant to β-lactam antibiotics, aminoglycosides, and macrolide-lincosamide-streptogramin B antibiotics (10, 17). Nearly all *S. haemolyticus* strains are susceptible to chloramphenicol (17). Multiple antibiotic resistance appears to be common in *S. haemolyticus* (9, 10, 17). However, the mechanisms of antimicrobial resistance and the localization of the resistance genes in *S. haemolyticus*, as well as the extent to which *S. haemolyticus* shares common resistance determinants with other staphylococcal species, are still unknown (17). In other coagulase-negative staphylococcal species from infections of humans and other animals, the determinants for antimicrobial resistance have often been found to be encoded by plasmids (17, 29–32). The existence of plasmids in *S. haemolyticus* has been demonstrated before, but their role in antibiotic resistance remains to be determined (16, 17).

We isolated *S. haemolyticus* from a urine specimen from a dog suffering from a urinary tract infection. This *S. haemolyticus* isolate was examined for its plasmid content and antimicrobial resistance pattern, with particular reference to chloramphenicol resistance (Cm^r). Although the use of chloramphenicol in humans is limited to a narrow range of selected infections, chloramphenicol is used in small animals, such as dogs and cats, to control a broad range of infections. The present report describes the isolation of a small Cm^r plasmid from *S. haemolyticus* as well as characterization of the *cat* gene and its gene product, an inducible chloramphenicol acetyltransferase (CAT).

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. haemolyticus* SH 227 was isolated from a urine specimen from a 7-year-old male German shepherd dog with a urinary tract infection. This staphylococcal isolate was identified as *S. haemolyticus* by the API-STAPH System (Bio Merieux, La Balme les Grottes, France) (3). The recipient strain for the plasmid transformation experiments, *S. aureus* RN 4220, was kindly provided by Michael Palmer, Institute for Medical Microbiology, Justus Liebig University, Giessen, Federal Republic of Germany. It was originally isolated by R. P. Novick, Public Health Institute, New York, N.Y.

Stock cultures of *S. haemolyticus* were prepared by incubation for 18 h on sheep blood agar plates (blood agar base [Oxoid, Basingstoke, United Kingdom] supplemented with 5% sheep blood) with subsequent storage at 4°C. Unless indicated otherwise, single colonies from the stock culture plates were grown in brain heart infusion broth (GIBCO, Paisley, Scotland) for 18 h at 37°C on a rotary shaker. Antibiograms were obtained from sulfonamide sensitivity test agar plates (Merck, Darmstadt, Federal Republic of Germany). Osmotically stable media for the protoplast transformation experiments were prepared as described previously (7).

Susceptibility testing. The *S. haemolyticus* isolate was tested for its antimicrobial susceptibility pattern by the agar diffusion method (2). Disks containing 20 μg of amoxicillin–10 μg of clavulanic acid, 25 μg of ampicillin, 30 μg of cephalothin, 30 μg of chloramphenicol, 10 μg of clindamycin, 10 μg of erythromycin, 10 μg of gentamicin, 30 μg of kanamycin, 10 μg of penicillin G, 10 μg of streptomycin, 23.75 μg of sulfamethoxazole, or 30 μg of tetracycline

(Oxoid) were used. The antibiograms were evaluated after incubation for 24 h at 37°C (2). The antimicrobial susceptibilities of the transformants were determined in the same manner.

Plasmid preparation. Plasmid DNA was isolated by a modification of the alkaline lysis procedure of Birnboim and Doly (4). The modification consisted of the removal of the staphylococcal cell walls by treatment with lysostaphin (Sigma, Deisenhofen, Federal Republic of Germany) at a final concentration of 40 μg/ml for 45 min at 37°C prior to alkaline denaturation (28, 29).

Plasmid transformation. A previously described modification (29) of the method of Chang and Cohen (7) was used for transfer of the *S. haemolyticus* plasmids. *S. aureus* RN 4220, a derivative of *S. aureus* 8325 (25), was chosen as a recipient since it was susceptible to all tested antibiotics and carried no plasmid. The transformed *S. aureus* protoplasts were selected on DM 3 regeneration plates (7) supplemented with 30 μg of chloramphenicol per ml. Clones which appeared on these selective media after 48 to 72 h were screened for plasmid DNA.

Restriction endonuclease mapping, cloning, and DNA sequencing. The restriction enzymes *Acc*I, *Bgl*II, *Bst*EII, *Cfo*I, *Cla*I, *Eco*RI, *Hin*dIII, *Hin*fI, *Hpa*II, *Mbo*I, *Pst*I, *Pvu*II, *Sac*I, *Taq*I, and *Xba*I were purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany, and used as recommended by the supplier. Standard DNA manipulation techniques were used. Cloning was carried out as described by Maniatis et al. (23). DNA sequencing was performed by the dideoxy chain termination method (26) as modified for double-stranded templates (23), using T7 DNA polymerase (39) and α-^{35}S-dATP (>600 Ci/mmol) from Amersham. DNA was sequenced on both strands, using oligonucleotide primers. A first set of primers was purchased from Stratagene, Heidelberg, Federal Republic of Germany. From the sequence data achieved with these primers, four synthetic 17-mer oligonucleotide primers were designed, synthesized with an Applied Biosystems 380 B DNA synthesizer, and used for sequence analyses (5).

Isolation of CAT. A previously described rapid colorimetric CAT assay (34) was used to demonstrate chloramphenicol inactivation by CAT-mediated transfer of acetyl groups from acetyl coenzyme A (acetyl-CoA) to chloramphenicol.

To induce *cat* gene expression, pSCS5 transformants were grown in brain heart infusion broth supplemented with chloramphenicol to a final subinhibitory concentration of 30 μg ml^{-1}. Since chloramphenicol itself served as a substrate for CAT, inactivated acetylated chloramphenicol had to be replaced by fresh chloramphenicol after each doubling of the bacteria. The pSCS5 transformants were grown in 5 liters of brain heart infusion broth to the mid-exponential phase (optical density at 600 nm, 0.65). After a final addition of chloramphenicol, the pSCS5 transformants were allowed to grow for another 30 min at 37°C to allow maximum *cat* expression. Cell-free lysates of the chloramphenicol-induced pSCS5 transformants were prepared as described previously (29, 34). CAT was precipitated from these cell-free lysates by the addition of ammonium sulfate in a final concentration of 70%, followed by stirring at 4°C for 12 h. The precipitate was collected by centrifugation (30 min, 4°C, 15,000 × *g*), resuspended in 20 ml of TCM buffer (50 mM Tris HCl [pH 7.8], 50 mM β-mercaptoethanol, 0.2 mM chloramphenicol), and dialyzed against the same buffer for 18 h. The CAT-containing solution was applied to a DEAE-cellulose column (3 by 10 cm, DE-52; Whatman Biochemical Ltd., Maidstone, United Kingdom) equilibrated with TCM buffer. The column was washed with TCM buffer, and CAT was eluted with a linear gradient of 0.0 to 0.4 M NaCl at a flow rate of 40 ml/h. Fractions of 5 ml were collected and tested for CAT activity, using the rapid CAT assay. The fraction from the ion-exchange chromatography with the highest CAT activity was applied in 200-μl samples to a fast protein liquid chromatography (FPLC) superose column (12 HR, 10/30; LKB, Freiburg, Federal Republic of Germany). The FPLC column had been equilibrated with 50 mM phosphate buffer (pH 7.3) supplemented with 100 mM sodium chloride. Elution profiles and times were monitored with a UV/VIS filter photometer (Knaur, Berlin, Germany) and a C-R3A chromatopac processor (Shimadzu, Kyoto, Japan). The fractions of the respective protein peaks were collected and tested for CAT activity. A 20-μl sample of the FPLC fraction with the highest CAT activity was analyzed in a 15% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel.

Protein was precipitated from the CAT solutions by the addition of an equal volume of 20% (wt/vol) trichloroacetic acid. After 16 h at 4°C, the precipitates were centrifuged and washed twice with 10% (wt/vol) trichloroacetic acid. The protein content was measured by the method of Lowry et al. (21), with bovine serum albumin (Serva, Heidelberg, Federal Republic of Germany) as a standard.

The M_r of native CAT was determined by FPLC with a superose column (12 HR, 10/30; LKB) at a flow rate of 30 ml/h, and the elution buffer consisted of 50 mM phosphate buffer (pH 7.3) supplemented with 50 mM sodium chloride. A calibration curve was constructed with the following proteins obtained from Serva: RNase A (M_r, 13,700), ovalbumin (M_r, 45,000), bovine serum albumin (M_r, 67,000), and aldolase (M_r, 160,000).

Isoelectric focusing. The fraction from ion-exchange chromatography with the highest CAT activity was dialyzed against distilled water for 24 h and then applied to a preparative isoelectric focusing column (110 ml; LKB, Stockholm, Sweden) under the conditions described by Schaeg et al. (27). The carrier ampholytes ranged from pH 3.5 to 10.0. Fractions of 1 ml were collected at a flow rate of 40 ml/h; their pH values were determined with an electronic pH meter (PHM 83; Radiometer, Copenhagen, Denmark), and their A_{280} was determined with an M4 Q III photometer (Zeiss, Oberkochen, Federal Republic of Germany). After dialysis against TCM buffer, the CAT activity of the fractions was determined by the rapid CAT assay.

Biochemical characterization of CAT. The Michaelis constants were determined by using spectrophotometric enzyme assays with FPLC-purified CAT solution (1 U/ml in 50 mM Tris HCl, pH 7.8) under saturating second-substrate conditions (100 μM chloramphenicol or 200 μM acetyl-CoA). First-substrate concentrations of 1.0 to 3.0 μM chloramphenicol as well as 10 to 70 μM acetyl-CoA were used. Double-reciprocal plots of velocity versus first-substrate concentrations, by the method of Lineweaver and Burk (19), were determined. Velocity of the enzyme reaction was expressed as production of CAT-dependent 5-thio-2-nitrobenzoate after 3 min at 37°C. The method of least squares was used to find the slope of the straight line.

The effect of pH on the activity of the pSCS5-encoded CAT was determined spectrophotometrically. CAT activity was tested at pH values of 6.8, 7.0, 7.3, 7.8, 8.0, and 8.3.

The influence of temperature on CAT activity was determined by incubation of 100-μl samples of the FPLC-purified CAT solution (1 U/ml) at 70°C in a water bath for 1, 3, 6, 9, 12, and 15 min and subsequent testing for CAT activity. A

100-μl sample of the same CAT solution, but not heat treated, served as a control for the original CAT activity.

The influence of mercury ions on CAT activity was determined as follows. DEAE-cellulose-purified CAT solution (3 U/ml) was dialyzed overnight at 4°C against 50 mM Tris HCl (pH 7.8) to eliminate resident chloramphenicol, which protects CAT against mercury ions. Of this dialyzed CAT solution, each 500 μl was supplemented with $HgCl_2$ at final concentrations of 0.1, 0.3, 0.5, and 1.0 mM, and subsequently the solution was incubated for 10 min at 37°C. Since mercury ions might interfere with the CAT assay, these probes were dialyzed again overnight at 4°C against 50 mM Tris HCl (pH 7.8). CAT activity was finally determined in the rapid CAT assay. The same CAT solution, also dialyzed twice but not supplemented with $HgCl_2$, was used to determine the original CAT activity.

Nucleotide sequence accession number. The sequence data reported here (see Fig. 2) have been submitted to GenBank and assigned the accession number M58515.

RESULTS

Antimicrobial resistance patterns and plasmid content. *S. haemolyticus* SH 227 proved to be resistant to amoxicillin-clavulanic acid, ampicillin, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, penicillin G, streptomycin, sulfamethoxazole, and tetracycline. It carried three small plasmids of 2.35, 3.75, and 4.6 kb. In interspecies protoplast transformation experiments, the 3.75-kb plasmid could be transferred to *S. aureus* RN 4220, in which it mediated Cm^r. This plasmid, designated pSCS5, was considered to carry the genetic information for Cm^r. The 4.6-kb plasmid carried the determinant for tetracycline resistance, and the 2.35-kb plasmid carried that for macrolide-lincosamide resistance.

Restriction endonuclease analyses. The Cm^r plasmid pSCS5 from *S. haemolyticus* was characterized by digestion with restriction endonucleases. A restriction map of pSCS5 is shown in Fig. 1. This restriction map served for structural comparisons between pSCS5 from "canine" *S. haemolyticus* and the prototype Cm^r plasmids pC221 (5, 11), pC223 (11), and pC194 (11, 15) from human *S. aureus* isolates. pSCS5 revealed structural similarities to the *rep* regions of pC221 and pC223. However, the regions encoding CAT in pC221 and pC194 differed significantly from the restriction map of pSCS5. Minor structural similarities in the regions encoding CAT were observed in the restriction maps of pC223 and pSCS5; both plasmids had single *Bst*EII and *Bgl*II cleavage sites in this region, but the flanking cleavage sites for restriction endonucleases differed significantly.

DNA and predicted amino acid sequences of the pSCS5-encoded *cat* gene. The structural *cat* gene as well as its regulatory region were located on two contiguous *Taq*I fragments of 0.90 and 0.64 kb. Both fragments were cloned in both directions into the single *Cla*I site of pBluescript $SKII^+$.

Sequence analyses revealed an open reading frame of 657 bp which encodes the structural *cat* gene. The respective regulatory region was located immediately upstream from the CAT coding region. This regulatory region consisted of two Shine-Dalgarno sequences (SD1, positions 125 to 134; SD2, positions 197 to 202), an open reading frame for a small peptide of nine amino acids (positions 143 to 169), and inverted repeats at positions 163 to 174 and 193 to 204. The DNA sequence and predicted amino acid sequence of the *cat* gene from pSCS5 are shown in Fig. 2. Comparisons of the

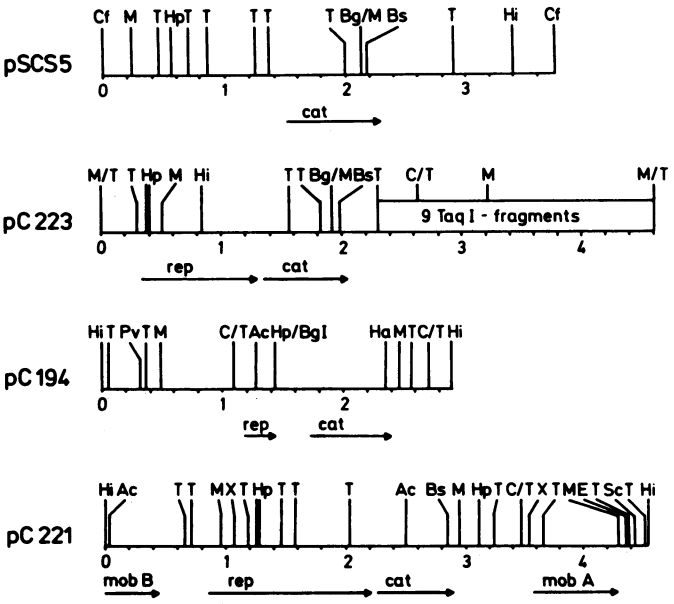

FIG. 1. Comparative analyses of the restriction maps of Cm^r plasmids pC221, pC223, and pC194 from *S. aureus* and pSCS5 from *S. haemolyticus*. Restriction endonuclease cleavage sites are as follows: Ac, *Acc*I; Bg, *Bgl*I; Bs, *Bst*EII; C, *Cla*I; Cf, *Cfo*I; E, *Eco*RI; Ha, *Hae*III; Hf, *Hin*fI; Hi, *Hin*dIII; Hp, *Hpa*II; M, *Mbo*I; Pv, *Pvu*II; Sc, *Sac*I; T, *Taq*I; X, *Xba*I. Distance scales in kilobases are shown below each map. The regions encoding replication functions (rep), CAT, or mobilization functions (mob A and mob B) are marked below the restriction maps; arrows indicate the direction of transcription of the *rep*, *cat*, and *mob* genes.

entire *cat* genes from pSCS5 and pC221 showed that these genes shared 81.3% DNA sequence identity and 75.3% amino acid sequence identity. However, the entire *cat* genes of pSCS5 and pC194 shared only 64.5% DNA sequence identity and 53.9% amino acid sequence identity. For better comparison of the pSCS5-encoded CAT with other CAT variants, an alignment of the predicted CAT amino acid sequences by the method of Hein (14) was performed (Fig. 3). This comparison revealed that the CAT variants encoded by the staphylococcal plasmids pSCS5, pC221, and pC194, i.e., CAT D from *Clostridium difficile* (4), CAT P from *C. perfringens* (38), CAT 86 from *Bacillus pumilis* (13), and type I CAT encoded by transposon Tn*9* (1), had only 44 amino acids in common.

Mechanism of chloramphenicol resistance. The pSCS5-transformed *S. aureus* and the original *S. haemolyticus* strain inactivated chloramphenicol by acetylation in the presence of acetyl-CoA. This could be shown in the rapid CAT assay. The pSCS5-encoded CAT was detectable by SDS-polyacrylamide gel electrophoresis (PAGE) only in cell-free lysates of chloramphenicol-induced pSCS5 transformants and not in those of uninduced pSCS5 transformants. Thus, pSCS5-encoded CAT was considered to be an inducible enzyme.

Purification and biochemical characteristics of the pSCS5-encoded CAT. The pSCS5-encoded CAT could be purified from the cell-free lysates of chloramphenicol-induced pSCS5 transformants by ammonium sulfate precipitation, followed by ion-exchange chromatography and FPLC. A specific CAT activity of 2.56 U/mg of protein was measured in the cell-free lysates. A 5.7-fold increase in specific CAT activity

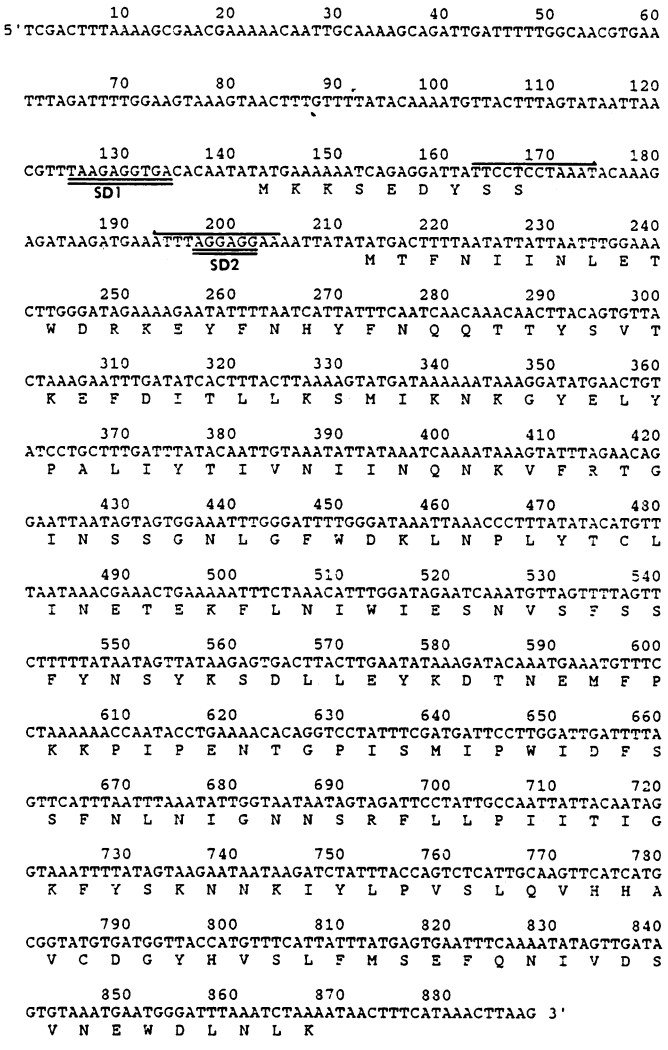

FIG. 2. DNA sequence of an 888-bp fragment containing the structural *cat* gene and its regulatory region, presented as the noncoding strand. Two putative Shine-Dalgarno sequences (SD1 and SD2) are marked. The arrows (→ ←) indicate the directions and the extent of the inverted repeats. The amino acid sequences of the two open reading frames, shown by the single-letter code, have been deduced from the respective nucleotide sequences.

to 14.51 U/mg of protein was found after purification by ion-exchange chromatography. The purified CAT from FPLC revealed a single band in SDS-PAGE (Fig. 4) and had a specific activity of 133.3 U/mg of protein.

The M_r of native CAT was determined by FPLC to be approximately 70,000. The subunit structure was examined by PAGE in the presence of 0.5% SDS. In SDS-PAGE the enzyme migrated as a single band of Coomassie blue-stained protein with an M_r of approximately 23,000. Thus, pSCS5-encoded CAT appeared to be a trimer composed of three identical subunits.

The isoelectric point of the purified enzyme was at pH 6.15. The K_m for chloramphenicol was calculated to be 2.81 μM, and that for acetyl-CoA was calculated to be 51.8 μM. Lineweaver-Burk diagrams were used for determination of K_m values.

The pSCS5-encoded CAT demonstrated optimal activity at a pH of 7.8 (Fig. 5). It also proved to be relatively heat stable since the remaining CAT activity was >80% of the original activity after 15 min at 70°C (Fig. 6). However, CAT activity was significantly reduced when mercury ions were present in the assay solution. A concentration of even 0.1 mM $HgCl_2$ led to a decrease in CAT activity of approximately 85% (Fig. 7).

DISCUSSION

Cm^r has often been found to be associated with the plasmid-encoded enzyme CAT, which inactivates chloramphenicol by acetylation in the presence of acetyl-CoA (33, 37). Two groups of CAT have been distinguished in *E. coli* and *S. aureus* (35). In *E. coli*, CAT was synthesized constitutively and encoded by large plasmids of >20 kb. These plasmids often also carried other resistance determinants, such as ampicillin resistance. In *S. aureus* strains from human infections, Cm^r had been found to be associated with small multicopy plasmids which range in size from 2.9 to 5.1 kb (22).

Three distinct families of Cm^r plasmids have been identified in *S. aureus* on the basis of restriction endonuclease analyses and DNA-DNA hybridization studies (11). The prototype plasmids of these three families are pC221, pC223, and pC194 (11). Recently, plasmid-encoded Cm^r was also detected in various staphylococcal species of animal origin. These Cm^r plasmids from canine *S. epidermidis* (32), canine *S. intermedius* (30), "porcine" *S. hyicus* (29), and "equine" *S. sciuri* (31) have been subjected to restriction endonuclease analyses. Their restriction maps revealed extensive structural homologies to the *rep/cat* region of pC221. Therefore, these plasmids were classified as new members of the pC221 family. However, the current study has shown that pSCS5 from *S. haemolyticus* is different from previously characterized staphylococcal Cm^r plasmids since its restriction map differed distinctly from those of pC221, pC223, and pC194. Despite the observed differences in the restriction maps, all staphylococcal Cm^r plasmids, including pSCS5, share a common resistance mechanism, namely, an inducible CAT. At least five variants of this enzyme have been distinguished in staphylococci; four of them were designated A, B, C, and D (8), and one encoded by plasmid pC194 was considered a fifth variant (35). No genetic analyses of CAT variants A, B, and D have been conducted (35, 36). So far, the *cat* genes of the *S. aureus* prototype plasmids pC221 and pC194 and their respective regulatory regions have been sequenced. The regulatory region in pSCS5 which is located 5′ of the CAT coding region contains all structural elements required for the attenuation regulatory model (6, 20). On mRNA transcribed from that regulatory region, the inverted repeats were able to form a stable stem-loop structure. This stem-loop was considered to act as a translation termination signal because the ribosome-binding site SD2 within the stem would not be accessible for ribosome binding. However, SD1 could interact with 16S rRNA, and translation of the following small open reading frame would be expected to open the stem-loop structure and render SD2 accessible for ribosomes. The small open reading frames for the nine-amino-acid peptide (MKKSEDYSS) were found to be identical in pC221 and pSCS5, but differed distinctly from the open reading frame for the respective six-amino-acid peptide (MKKADK) in pC194. While the inverted repeats in pC221, pC194, and pSCS5 exhibited the same length of 12 nucleotides each, the SD1 sequences of these plasmids differed

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CAT pSCS5     MT-----FNIINLETWDRKEYFNHYFNQQT-TYSVTKEFDITLLKSMIKNKGYELYPALI  54
CAT pC221     MT-----FNIIKLENWDRKEYFEHYFNQQT-TYSITKEIDITLFKDMIKKKGYEIYPSLI  54
CAT pC194     MN-----FNKIDLDNWKRKEIFNHYLNQQT-TFSITTEIDISVLYRNIKQEGYKFYPAFI  54
CAT D         MV-----FEKIDKNSWNRKEYFDHYFASVPCTYSMTVKVDITQ----IKEKGMKLYPAML  51
CAT P         MV-----FEKIDKNSWNRKEYFDHYFASVPCTYSMSLKVDITQ----IKEKGMKLYPAML  51
CAT 86        M------FKQIDENYL-RKEHFHHYMTLTRCSYSLVINLDITKLHAILKEKKLKVYPVQI  53
TypeI CAT     MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKFYPAFI  60
              *                *** *                  **       *      **

CAT pSCS5     YTIVNIINQNKVFRTGINSSGNLGFWDKLNPLYTCLINETEKFLNIWIESNVSFSSFYNS  113
CAT pC221     YAIMEVVNKNKVFRTGINSENKLGYWDKLNPLYTVFNKQTEKFTNIWTESDNNFTSFYNN  113
CAT pC194     FLVTRVINSNTAFRTGYNSDGELGYWDKLEPLYTIFDGVSKTFSGIWTPVKNDFKEFYDL  113
CAT D         YYIAMIVNRHSEFRTAINQDGELGIYDEMIPSYTIFHNDTETFSSLWTECKSDFKSFLAD  110
CAT P         YYIAMIVNRHSEFRTAINQDGELGIYDEMIPSYTIFHNDTETFSSLWTECKSDFKSFLAD  110
CAT 86        YLLARAVQKIPEFRMDQVND-ELGYWEILHPSYTILNKETKTFSSIWTPFDENFAQFYKS  112
TypeI CAT     HILARLMNAHPEFRMAMK-DGELVIWDSVHPCYTVFHEQTETFSSLWSEYHDDFRQFLHI  119
                       **       *       * **       *   *      *  *

CAT pSCS5     YKSDLLEYKDTNEMFPKKPIPENTGPISMIPWIDFSSFNLNIGNNSRFLLPIITIGKFYS  172
CAT pC221     YKNDLLEYKDKEEMFPKKPIPENTIPISMIPWIDFSSFNLNIGNNSNFLLPIITIGKFYS  172
CAT pC194     YLSDVEKYNGSGKLFPKTPIPENAFSLSIIPWTSFTGFNLNINNNSNYLLPIITAGKFIN  172
CAT D         YESDTQRYGNNHRMEGKPNAPENIFNVSMIPWSTFDGFNLNLQKGYDYLIPIFTMGKIIK  169
CAT P         YESDTQRYGNNHRMEGKPNAPENIFNVSMIPWSTFDGFNLNLQKGYDYLIPIFTMGKIIK  169
CAT 86        CVADIETFSKSSNLFPKPHMPENMFNISSLPWIDFTSFNLNVSTDEAYLLPIFTIGKFKV  171
TypeI CAT     YSQDVACYGENLAYFPKGFI-ENMFFVSANPWVSFTSFDLNVANMDNFFAPVFTMGKYYT  177
                 *            *    **    *  **  *  * **         *  * **

CAT pSCS5     KNNKIYLPVSLQVHHAVCDGYHVSL-------FMSEFQNIVDSVNEWDLNLK-------  219
CAT pC221     ENNKIYIPVALQLHHAVCDGYHASL-------FMNEFQDI--------IH-K--VDDWI  215
CAT pC194     KGNSIYLPLSLQVHHSVCDGYHAGL-------FMNSIQEL--------SDRP--NDWLL  216
CAT D         KDNKIILPLAIQVHHAVCDGFHICR-------FVNELQEL--------II-V--TQVCL  212
CAT P         KDNKIILPLAIQVHHAVCDGFHICR-------FVNELQEL--------II-V--TQVCl  212
CAT 86        EEGKIILPVAIQVHHAVCDGYHAGQYVEYLRWLIEHCDEW--------LN-D--SLHIT  220
TypeI CAT     QGDKVLMPLAIQVHHAVCDGFHVGR-------MLNELQQY--------CD-EWQGG--A  219
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FIG. 3. Alignment of the predicted CAT amino acid sequences by the method of Hein (14). Abbreviations: CAT pSCS5, CAT encoded by *S. haemolyticus* plasmid pSCS5; CAT pC221, CAT encoded by *S. aureus* plasmid pC221; CAT pC194, CAT encoded by *S. aureus* plasmid pC194; CAT D, chromosomally encoded CAT from *C. difficile*; CAT P, CAT encoded by *C. perfringens* plasmid pIP401; CAT 86, chromosomally encoded CAT from *B. pumilis*; type I CAT, CAT encoded by transposon Tn9. Asterisks indicate conserved amino acids in all CAT sequences.

distinctly from one another. mRNA transcribed from these SD1 sequences demonstrated a high degree of sequence complementarity to *B. subtilis* 16S rRNA. The free energy for this mRNA-rRNA interaction was calculated (40) to be −18.6 kcal (ca. −77.8 kJ)/mol for pC221 (6), −18.2 kcal (ca. −76.2 kJ)/mol for pSCS5, and −11.8 kcal (ca. −49.3 kJ)/mol for pC194 (6).

Furthermore, comparisons of the structural *cat* genes from pC221, pC194, and pSCS5 showed that the three *cat* genes differed slightly in their sizes: *cat* from pC221 consisted of 645 bp; *cat* from pC194, 648 bp; and *cat* from pSCS5, 657 bp. These three staphylococcal *cat* genes also differed distinctly in their nucleotide sequences and their predicted amino acid sequences. The amino acid alignment

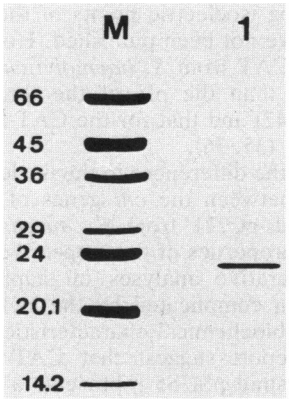

FIG. 4. Coomassie blue-stained gel after SDS-PAGE of FPLC-purified CAT from pSCS5 (lane 1). Lane M contains the marker proteins (M_r, 10^3): bovine serum albumin (M_r, 66,000); ovalbumin (M_r, 45,000); glutaraldehyde 3-phosphate dehydrogenase (M_r, 36,000); carbonic anhydrase (M_r, 29,000); trypsinogen, phenylmethylsulfonyl fluoride treated (M_r, 24,000); trypsin inhibitor (M_r, 20,100); and α-lactalbumin (M_r, 14,200).

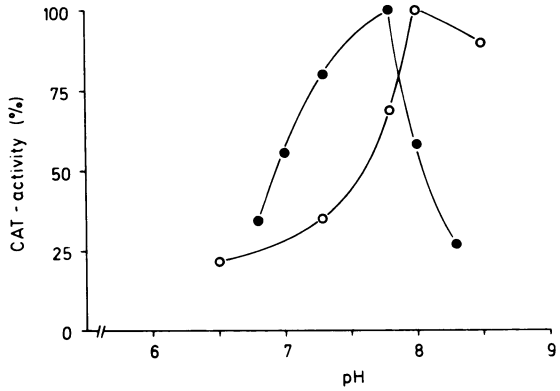

FIG. 5. Influence of pH on CAT activity. ●, activity of pSCS5-encoded CAT; ○, activity of pC221-encoded CAT (31). Values are expressed as percentage of maximum CAT activity.

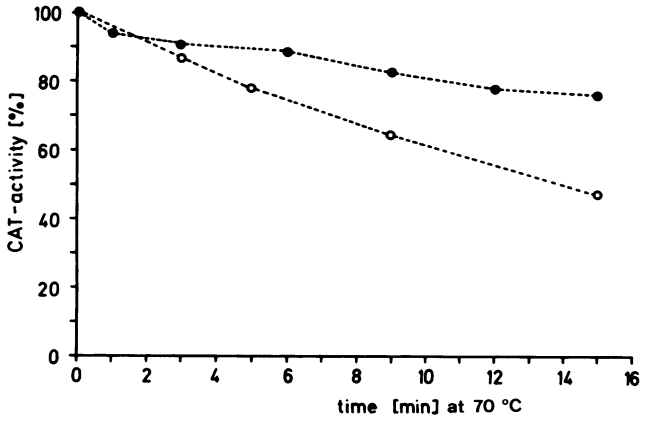

FIG. 6. Influence of heat treatment at 70°C on CAT activity. ●, activity of pSCS5-encoded CAT; ○, activity of pC221-encoded CAT (31). Values are expressed as percentage of activity of the unheated control.

(Fig. 3) revealed the presence of 44 conserved residues in the CAT variants from *Staphylococcus* spp., *Clostridium* spp., *B. pumilis*, and *E. coli*. This suggested a high degree of amino acid variability within the CAT monomers found in different bacterial species. However, the active center of CAT (His-186) as well as the respective flanking amino acids were highly conserved not only in the staphylococcal CAT variants but also in those of other gram-positive and gram-negative bacteria. Despite the observed differences in the predicted CAT amino acid sequences, all CAT variants exhibited the same quarternary structure. For more than a decade, all CAT variants were considered to exist as tetramers, consisting of four identical subunits. However, more recent X-ray crystallography of the R-factor-encoded type III CAT from *Shigella flexneri* revealed a trimeric structure (18). Such a trimeric structure has also been suggested for staphylococcal CAT variants (29). Our data on pSCS5-encoded CAT from *S. haemolyticus* are in accordance with this observation. The M_r of native CAT from pSCS5, approximately 70,000, corresponded closely to that of the sum of the three subunits. The M_r of one subunit was calculated

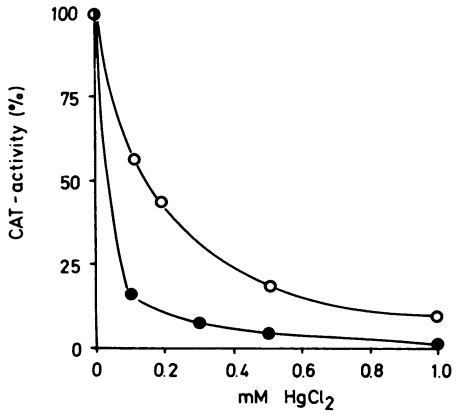

FIG. 7. Influence of mercury ions on CAT activity. ●, activity of pSCS5-encoded CAT; ○, activity of pC221-encoded CAT (37). Enzyme activity is expressed as percentage of activity of the untreated control.

TABLE 1. Properties of CAT variants from different bacterial species

Bacterial species and enzyme type	Plasmid	K_m (μM)		Isoelectric point (pH)	Reference
		Chloramphenicol	Acetyl-CoA		
S. aureus					
A		2.6	57		8
B		2.7	56		8
C	pC221	2.5	61		8
D		2.7	47		8
None	pC194				35
S. haemolyticus	pSCS5	2.8	52	6.15	This paper
E. coli					
I	R1	11.5	76	5.40	36
II	R753	18.0	57	5.10	36
III	R387	16.3	80	4.80	36
A. tumefaciens		20.5	133	3.90	36

to be 23,000. This M_r is also very similar to the reported value of 22,500 for CAT encoded by pC221 (42).

Fairly subtle differences in the K_m values for Cm and acetyl-CoA were observed between the pSCS5-encoded CAT and the previously investigated staphylococcal CAT variants (Table 1). Since only CAT variant C, encoded by plasmid pC221, had been studied in detail for its further biochemical and enzymological properties, comparisons concerning pH optima, heat stability, and sensitivity to mercury ions were limited to the CAT variants from pSCS5 and pC221. CAT from pSCS5 exhibited the highest activity at pH 7.8; CAT from pC221 exhibited the highest activity at pH 8.0. However, differences in the pH curves of both CAT variants could be observed (Fig. 5). CAT from pSCS5 proved to be more resistant to heat treatment since its residual activity after 15 min at 70°C was >1.5-fold higher than the residual activity of CAT from pC221 under the same conditions. Both CAT variants were also very sensitive to $HgCl_2$ at concentrations of 1.0 mM. However, 10-fold-lower $HgCl_2$ concentrations revealed significantly different decreases in activities of both enzymes. CAT from pSCS5 exhibited a decrease in activity of approximately 85% at a concentration of 0.1 mM $HgCl_2$, whereas CAT from pC221 demonstrated a residual activity of >50% at the same $HgCl_2$ concentration.

Data concerning isoelectric points of the staphylococcal CAT variants have not been published. However, the pI of pSCS5-encoded CAT from *S. haemolyticus* (pH 6.15) was distinctly higher than the pIs of the three *E. coli* CAT variants (35, 36, 42) and that for the CAT from *Agrobacterium tumefaciens* (35, 36).

In conclusion, the differences in the nucleotide and amino acid sequences between the *cat* genes of pSCS5 from *S. haemolyticus* and pC221 from *S. aureus* seemed to be reflected by the properties of the respective resistance gene products. Comparative analyses of staphylococcal CAT variants had been complicated by the lack of information concerning their biochemical characteristics. The data presented in this report suggest that CAT encoded by *S. haemolyticus* plasmid pSCS5 represents a sixth CAT variant, which is not identical to the CAT variants encoded by pC221 and pC194.

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REFERENCES

1. **Alton, N. K., and D. Vapnek.** 1979. Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. Nature (London) **282**:864–869.
2. **Barry, A. L., and C. Thornsberry.** 1985. Susceptibility tests: diffusion test procedures, p. 978–987. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
3. **Bio Merieux.** 1988. API STAPH catalogue analytique, 2nd ed. Bio Merieux, La Balme les Grottes, France.
4. **Birnboim, H. C., and J. Doly.** 1978. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Brenner, D. G., and W. V. Shaw.** 1985. The use of synthetic oligonucleotides with the universal templates for rapid DNA sequencing: results with staphylococcal replicon pC 221. EMBO J. **4**:561–568.
6. **Brückner, R., and H. Matzura.** 1985. Regulation of the inducible chloramphenicol acetyltransferase gene of the *Staphylococcus aureus* plasmid pUB 112. EMBO J. **4**:2295–2300.
7. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
8. **Fitton, J. E., and W. V. Shaw.** 1979. Comparison of chloramphenicol acetyltransferase variants in staphylococci. Biochem. J. **177**:575–582.
9. **Fleurette, J., Y. Brun, M. Bes, M. Coulet, and F. Forey.** 1987. Infections caused by coagulase-negative Staphylococci other than *S. epidermidis* and *S. saprophyticus*. Zentralbl. Bakteriol. Suppl. 16.
10. **Frogatt, J. W., J. L. Johnston, D. W. Galetto, and G. L. Archer.** 1989. Antimicrobial resistance in nosocomial isolates of *Staphylococcus haemolyticus*. Antimicrob. Agents Chemother. **33**: 460–466.
11. **Gillespie, M. T., and R. A. Skurray.** 1988. Structural relationships among chloramphenicol-resistance plasmids of *Staphylococcus aureus*. FEMS Microbiol. Lett. **51**:205–210.
12. **Gunn, B. A., and C. E. Davis, Jr.** 1988. *Staphylococcus haemolyticus* urinary tract infection in a male patient. J. Clin. Microbiol. **26**:1055–1057.
13. **Harwood, C. R., D. M. Williams, and P. S. Lovett.** 1983. Nucleotide sequence of a *Bacillus pumilis* gene specifying chloramphenicol acetyltransferase. Gene **24**:163–169.
14. **Hein, J.** 1990. Unified approach to alignment and phylogenies. Methods Enzymol. **183**:626–645.
15. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol. **150**:815–825.
16. **Kloos, W. E., B. S. Orban, and D. D. Walker.** 1981. Plasmid composition of staphylococcus species. Can. J. Microbiol. **27**:271–278.
17. **Kloos, W. E., and K. H. Schleifer.** 1986. Genus IV. *Staphylococcus*, p. 1013–1035. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
18. **Leslie, A. G. W., P. C. E. Moody, and W. V. Shaw.** 1988. Structure of chloramphenicol acetyltransferase at 1.75 A resolution. Proc. Natl. Acad. Sci. USA **85**:4133–4137.
19. **Lineweaver, H., and D. Burk.** 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.
20. **Lovett, P. S.** 1990. Translation attenuation as the regulator of inducible *cat* genes. J. Bacteriol. **172**:1–6.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **Lyon, B. R., and R. Skurray.** 1987. Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. Microbiol. Rev. **51**:88–134.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Nicolle, L. E., S. H. Hoban, and G. K. M. Harding.** 1983. Characterization of coagulase-negative staphylococci from urinary tract specimens. J. Clin. Microbiol. **17**:267–271.
25. **Novick, R. P.** 1967. Properties of a cryptic high frequency transducing phage in *Staphylococcus aureus*. Virology **33**:155–166.
26. **Sanger, F., S. Nicklen, and A. Coulsen.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
27. **Schaeg, W., R. Bingöl, and H. Blobel.** 1972. Purification of penicillinase (β-lactamase) and acid phosphatase from *Staphylococcus aureus* in one procedure. Biochim. Biophys. Acta **268**:542–549.
28. **Schwarz, S., and H. Blobel.** 1989. Plasmids and resistance to antimicrobial agents and heavy metals in *Staphylococcus hyicus* from pigs and cattle. J. Vet. Med. B **36**:669–673.
29. **Schwarz, S., M. Cardoso, and H. Blobel.** 1989. Plasmid-mediated chloramphenicol resistance in *Staphylococcus hyicus*. J. Gen. Microbiol. **135**:3329–3336.
30. **Schwarz, S., M. Cardoso, and H. Blobel.** 1989. Plasmid-encoded resistance to chloramphenicol in "canine" *Staphylococcus intermedius*-isolates. Med. Sci. Res. **17**:451–453.
31. **Schwarz, S., M. Cardoso, and H. Blobel.** 1990. Detection of a novel chloramphenicol resistance plasmid from "equine" *Staphylococcus sciuri*. J. Vet. Med. B **37**:674–679.
32. **Schwarz, S., M. Cardoso, S. Grölz-Krug, and H. Blobel.** 1990. Common antibiotic resistance plasmids in *Staphylococcus aureus* and *Staphylococcus epidermidis* from human and canine infections. Zentralbl. Bakteriol. Hyg. Reihe A **273**:369–377.
33. **Shaw, W. V.** 1967. The enzymatic acetylation of chloramphenicol by extracts of R-factor resistant *Escherichia coli*. J. Biol. Chem. **242**:687–693.
34. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant *Staphylococcus aureus*. Methods Enzymol. **43**:737–755.
35. **Shaw, W. V.** 1983. Chloramphenicol acetyltransferase: enzymology and molecular biology. Crit. Rev. Biochem. **14**:1–46.
36. **Shaw, W. V.** 1984. Bacterial resistance to chloramphenicol. Br. Med. Bull. **40**:36–41.
37. **Shaw, W. V., D. W. Bently, and L. Sands.** 1970. Mechanism of chloramphenicol resistance in *Staphylococcus epidermidis*. J. Bacteriol. **104**:1095–1103.
38. **Steffen, C., and H. Matzura.** 1989. Nucleotide sequence of a chloramphenicol-acetyltransferase coding gene from *Clostridium perfringens*. Gene **75**:349–354.
39. **Tabor, S., and G. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84**:4767–4771.
40. **Tinoco, I., Jr., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246**:40–41.
41. **Wren, B. W., P. Mullany, C. Clayton, and S. Tabaqchali.** 1989. Nucleotide sequence of a chloramphenicol acetyltransferase gene from *Clostridium difficile*. Nucleic Acids Res. **17**:4877–4877.
42. **Zaidenzaig, Y., J. E. Fitton, L. C. Packman, and W. V. Shaw.** 1979. Characterization and comparison of chloramphenicol acetyltransferase variants. Eur. J. Biochem. **100**:609–618.