Characterization of the chloramphenicol acetyltransferase variants encoded by the plasmids pSCS6 and pSCS7 from *Staphylococcus aureus*

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The two 4·6 kb chloramphenicol resistance (Cm^{R}) plasmids pSCS6 and pSCS7, previously identified in *Staphylococcus aureus* from subclinical bovine mastitis, both encoded an inducible chloramphenicol acetyltransferase (CAT, EC 2.3.1.28). The pSCS6- and pSCS7-encoded CAT variants were purified by ammonium sulphate precipitation, ion-exchange chromatography and fast protein liquid chromatography (FPLC). Both native enzymes showed M_r values of 70 000 on FPLC and were composed of three identical subunits, each of M_r approximately 23 000. The CAT variants from pSCS6 and pSCS7 differed in their net charges and in their isoelectric points. The isoelectric point of the CAT from pSCS6 was pH 5·7 and that of the CAT from pSCS7 pH 5·2. Both CAT variants exhibited highest enzyme activities at pH 8·0. The K_m values for chloramphenicol and acetyl-CoA of the CAT from pSCS6 were 2·5 μM and 58·8 μM , respectively, while those of the CAT from pSCS7 were 2·7 μM and 55·5 μM . Both CAT variants were relatively thermostable. The CAT from pSCS6 was less sensitive to mercuric ions than the CAT from pSCS7.

Introduction

Staphylococcus aureus is one of the major bacterial pathogens associated with bovine mastitis (Baumgartner *et al.*, 1984). Antibiotics are often used to control *S. aureus* infections (Blobel & Brückler, 1980), and this has led to *S. aureus* isolates becoming increasingly resistant to antimicrobial agents. In *S. aureus* isolates from infections of humans, several antibiotic-resistance genes have been found to be located on plasmids (Lacey, 1975; Lyon & Skurray, 1987). Chloramphenicol resistance (Cm^{R}) is exclusively encoded by small non-conjugative plasmids (Lyon & Skurray, 1987). Several Cm^{R} plasmids have been isolated from *S. aureus* of human origin and their structure and properties investigated (Gillespie & Skurray, 1988).

Although the existence of plasmids in *S. aureus* of bovine origin has been demonstrated before, their role in antibiotic resistance remains to be determined (Baumgartner *et al.*, 1984). Recently, four small Cm^{R} plasmids were isolated from bovine *S. aureus* (Cardoso & Schwarz, 1992a). The chloramphenicol acetyltransferase (*cat*)

genes of two of these plasmids, pSCS6 and pSCS7, have been sequenced, and differences were noted both in their nucleotide sequences and in their predicted CAT amino acid sequences (Schwarz & Cardoso, 1991b; Cardoso & Schwarz, 1992b).

The purpose of this investigation was to determine whether the differences in the nucleotide sequences of the pSCS6- and pSCS7-encoded *cat* genes could be associated with differences in the biochemical properties of the respective gene products. The CATs were purified and their subunit composition, their isoelectric points, their pH optima, their K_m values for chloramphenicol and acetyl-CoA, their thermostability and their sensitivity to mercuric ions were compared.

Methods

Bacterial strains, plasmids and growth conditions. The Cm^{R} plasmids pSCS6 and pSCS7 had previously been transferred into *S. aureus* RN4220 (Kreiwirth *et al.*, 1983; Cardoso & Schwarz, 1992a). These transformants were used to isolate the pSCS6- and pSCS7-encoded CAT variants. Single colonies from the stock culture plates were grown in Brain Heart Infusion broth (BHI, Gibco) for 18 h at 37 °C on a rotary shaker (160 r.p.m.).

Enzymic inactivation of chloramphenicol by acetylation. A previously described bioassay was used to demonstrate enzymic inactivation of

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Abbreviations: CAT, chloramphenicol acetyltransferase.

chloramphenicol (Schwarz *et al.*, 1989). Chloramphenicol acetyltransferases (CATs) were responsible for Cm^R as revealed by the rapid CAT assay (Shaw, 1975).

Isolation of CAT. (a) *Induction of CAT.* Expression of staphylococcal CAT variants is inducible (Lyon & Skurray, 1987). Induction of CAT is achieved with chloramphenicol (Winshell & Shaw, 1969), but since chloramphenicol is itself a substrate for CAT, the inducer is rapidly inactivated, leading to reduced induction rates. In order to optimize CAT induction, acetylated chloramphenicol had to be replaced by fresh chloramphenicol after each doubling of the RN4220(pSCS6) and RN4220(pSCS7) cultures to a final concentration of 50 µg ml⁻¹. The transformants were grown in 50 ml BHI to an OD₆₀₀ of 0.65. After a final addition of chloramphenicol, the cultures were grown for a further 30 min at 37 °C. The cells were then harvested by centrifugation (20 min, 4 °C, 6000 g).

To confirm the inducibility of the pSCS6- and pSCS7-encoded CAT variants, cultures were grown in the same manner, except that the medium was not supplemented with chloramphenicol.

(b) *Cell-free lysates and SDS-PAGE.* The harvested cells were washed with 5 ml TS buffer (50 mM-Tris/HCl and 145 mM-NaCl, pH 7.5). After resuspending the bacteria in 1 ml of the same buffer, lysostaphin (80 µg ml⁻¹) was added. After 15 min at 37 °C, DNAase (20 µg ml⁻¹) was added, followed by 15 min incubation at 37 °C. The supernatant obtained after centrifugation (20 min, 4 °C, 15000 g) was analysed by SDS-PAGE using 11% (w/v) polyacrylamide gels (Maniatis *et al.*, 1982) at 10 V cm⁻¹ for 6 h (Laemmli, 1970).

(c) *Purification of CAT.* Lysates of cells harvested from 5 litres of chloramphenicol-induced RN4220(pSCS6) and RN4220(pSCS7) cultures were prepared. CAT was precipitated by the addition of ammonium sulphate to 70% saturation, followed by stirring at 4 °C for 12 h. The precipitate was collected by centrifugation (30 min, 4 °C, 15000 g), resuspended in 20 ml TCM buffer (50 mM-Tris/HCl pH 7.8, 50 mM-mercaptoethanol, 0.2 mM-chloramphenicol) and dialysed against the same buffer for 18 h. Then the CAT-containing solution was applied to a DEAE-cellulose column (3 × 10 cm, DE-52, Whatman) equilibrated with TCM buffer. The column was washed with TCM buffer and CAT was eluted with a linear gradient of 0 to 0.4 M-NaCl at a flow rate of 40 ml h⁻¹. Fractions of 5 ml were collected and tested for CAT activity using the rapid CAT assay (Shaw, 1975).

The ion-exchange chromatography fraction with the highest CAT activity was applied in 200 µl samples to a Superose FPLC column (12 HR, 10/30, LKB) which had been equilibrated with 50 mM-sodium phosphate buffer (pH 7.3) supplemented with 100 mM-NaCl. Elution profiles and times were monitored with a Knauer UV/VIS filter photometer and a Shimadzu C-R3A chromatopac processor. 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 finally analysed by 15% (w/v) SDS-PAGE.

Electrophoresis of the native CAT variants was carried out in 1% (w/v) agarose gels in 65 mM-sodium barbital buffer (Shaw *et al.*, 1970). Agarose gels were run at running-buffer pH values of 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 for 2 h at 12 V cm⁻¹. Proteins were detected by staining with Coomassie Brilliant Blue.

Protein determinations. Protein was precipitated by the addition of an equal volume of 20% (w/v) trichloroacetic acid (TCA). After 16 h at 4 °C the precipitates were centrifuged and washed twice with 10% (w/v) TCA. The protein content was measured by the Lowry method, with bovine serum albumin (Serva) as a standard.

M_r determination of native CAT. 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⁻¹ and an elution buffer consisting of 50 mM-sodium phosphate (pH 7.3) and 50 mM-NaCl. A calibration curve was constructed with the following proteins, obtained from Serva: RNAase

A (M_r 13700), ovalbumin (45000), bovine serum albumin (67000) and aldolase (160000).

Isoelectric focussing. The ion-exchange chromatography fraction with the highest CAT activity was dialysed against distilled water for 24 h and then applied to a preparative isoelectric focussing column (110 ml, LKB) under the conditions described by Schaege *et al.* (1972). The carrier ampholytes ranged from pH 3.5 to 10.0. Fractions of 1 ml were collected and investigated for pH and CAT activity as previously described (Schwarz & Cardoso, 1991a).

Enzyme kinetics. The Michaelis constants were determined using spectrophotometric enzyme assays under saturating second-substrate conditions (100 µM-chloramphenicol or 200 µM-acetyl-CoA) (Fitton & Shaw, 1979). For these assays, the FPLC-purified CAT solution (1 U ml⁻¹ in 50 mM-Tris/HCl pH 7.8) was diluted 1000-fold. First-substrate concentrations of 1.0, 1.5, 2.0, 2.5 and 3.0 µM-chloramphenicol, and 10, 20, 30, 40, 50, 60 and 70 µM-acetyl-CoA, were used. Double reciprocal plots of velocity versus first-substrate concentrations according to Lineweaver & Burk (1934) were prepared. The velocity of the enzyme reaction was expressed as production of CAT-dependent 5-thio-2-nitrobenzoate after 1 min and 3 min at 37 °C. The assays were linear over the entire 3 min period. All measurements were performed in triplicate for each concentration of substrate. The method of least squares was used to find the slope of the straight line.

Influence of pH on CAT activity. The effect of pH on the activity of the pSCS6- and pSCS7-encoded CAT variants was determined spectrophotometrically, assaying 10 µl samples of a DEAE-cellulose-purified CAT solution (3 U ml⁻¹) at pH values of 6.0, 6.5, 7.0, 7.5, 7.8, 8.0 and 8.5.

Influence of temperature on CAT activity. Samples (100 µl) of the FPLC-purified CAT solution (1 U ml⁻¹) were heated at 70 °C in a water bath for 1, 3, 6, 9, 12 and 15 min and subsequently tested for their CAT activity. A 100 µl sample of the same CAT solution, but not heat-treated, served as a control for the original CAT activity.

Influence of mercuric ions on CAT activity. The DEAE-cellulose-purified CAT solution (3 U ml⁻¹) was dialysed overnight at 4 °C against 50 mM-Tris/HCl (pH 7.8) to eliminate residual chloramphenicol, which protects CAT against mercuric ions. Samples (500 µl) were supplemented with HgCl₂ to 0.1, 0.3, 0.5 and 1.0 mM and subsequently incubated for 10 min at 37 °C. Since mercury ions might interfere with the CAT assay, the samples were then dialysed again overnight at 4 °C against 50 mM-Tris/HCl (pH 7.8). CAT activity was finally determined by the rapid CAT assay. The same CAT solution, also dialysed twice but not supplemented with HgCl₂, was used to determine the original CAT activity.

Results

Mechanism of chloramphenicol resistance

Enzymic inactivation of chloramphenicol by the RN4220(pSCS6) and RN4220(pSCS7) transformants was demonstrated by bioassay. The enzymes were shown to inactivate chloramphenicol by acetylation in the presence of acetyl-CoA by the rapid CAT assay. Monomers of the pSCS6- and pSCS7-encoded CAT variants were detectable in SDS-PAGE of lysates of chloramphenicol-induced cells, but not in those of

Fig. 1. SDS-PAGE of cell-free lysates of chloramphenicol-induced (lanes 1) and un-induced (lanes 2) pSCS6 (a) and pSCS7 (b) transformants. Lanes M contain protein markers. The CAT monomers are indicated by arrowheads.

uninduced cells (Fig. 1). Thus, both CAT variants were considered to be inducible.

Purification of CAT

The pSCS6- and pSCS7-encoded CAT variants were purified by ammonium sulphate precipitation followed by ion-exchange chromatography. The CATs differed in their elution profiles on ion-exchange chromatography: while the CAT from pSCS6 could be eluted at 52 mM-NaCl, the CAT from pSCS7 was eluted at 74 mM-NaCl. FPLC allowed purification to homogeneity as indicated by SDS-PAGE analysis, which revealed single bands of M_r 23000 (Fig. 2).

Table 1 shows the results of the purification procedures. The purified CAT variants differed slightly in their specific activities: 280 U (mg protein)⁻¹ for the CAT from pSCS6, and 240 U (mg protein)⁻¹ for the CAT from pSCS7.

M_r and subunit structure

The M_r values of the native CAT variants were determined by FPLC to be approximately 70000 in each case (results not shown). On SDS-PAGE, both enzymes appeared as a single band of M_r 23000 (Fig. 2). Thus, the pSCS6- and pSCS7-encoded CAT variants appeared to be trimers composed of three identical subunits, each with an M_r of 23000.

Fig. 2. SDS-PAGE of the FPLC-purified CAT variants from pSCS6 (lane 1) and pSCS7 (lane 2). Lane M contains protein markers.

Isoelectric point and enzyme kinetics

The purified CATs differed in their isoelectric points. These were pH 5.7 for the CAT from pSCS6 and pH 5.2 for the CAT from pSCS7 (Table 2). Moreover, agarose gel electrophoresis of the non-denatured proteins demonstrated differences in the net charge. The CATs differed in their anodal migration patterns at varying pH values with respect to their isoelectric points (Fig. 3).

Table 1. Purification of the CAT variants encoded by the plasmids pSCS6 and pSCS7 from *S. aureus*

CAT encoded by	Purification step	Total activity (units ml ⁻¹)	Total protein (mg ml ⁻¹)	Specific activity [units (mg protein) ⁻¹]	Purification factor
pSCS6	Cell-free lysate	2.5	3.600	0.7	1.0
	Ion-exchange chromatography	16.2	1.760	9.2	13.1
	FPLC	7.0	0.025	280.0	400.0
pSCS7	Cell-free lysate	2.5	2.700	0.9	1.0
	Ion-exchange chromatography	11.0	1.170	9.4	10.4
	FPLC	6.0	0.025	240.0	266.7

Table 2. Properties of staphylococcal CAT variants

Staphylococcal species and enzyme type	Plasmid	K_m (μ M)		Isoelectric point	pH optimum	Heat stability	Reference(s)
		Chloramphenicol	Acetyl-CoA				
<i>S. aureus</i>							
A	—	2.6	57	—	—	Unstable	Fitton & Shaw (1979)
C	pC221	2.5	61	—	8.0	Stable	Shaw (1975) Fitton & Shaw (1979)
D	—	2.7	47	—	—	Stable	Shaw <i>et al.</i> (1970) Fitton & Shaw (1979)
—	pC194	—	—	—	—	—	Shaw (1975)
—	pSCS6	2.5	59	5.70	8.0	Stable	Shaw (1983) This paper
—	pSCS7	2.7	56	5.20	8.0	Stable	This paper
<i>S. epidermidis</i>							
B	—	2.7	56	—	8.0	Stable	Shaw <i>et al.</i> (1970) Shaw (1975)
<i>S. haemolyticus</i>	pSCS5	2.8	52	6.15	7.8	Stable	Schwarz & Cardoso (1991a)

—, No data available.

The K_m values for chloramphenicol and acetyl-CoA of CAT from pSCS6 were 2.5 μ M and 58.8 μ M, respectively; those of CAT from pSCS7 were 2.7 μ M and 55.5 μ M (Table 2).

Effects of pH, temperature and mercuric ions on CAT activity

Both CAT variants demonstrated highest activity at pH 8.0 (Table 2). They also proved to be relatively thermostable since the CAT activities remaining after 15 min at 70 °C were approximately 60% (pSCS6) and 55% (pSCS7) of the original activity. However, CAT activity was significantly reduced by the presence of mercuric ions (Fig. 4). While HgCl₂ concentrations of 0.1 and 0.3 mM had no influence on the activity of the pSCS6-encoded CAT, the activity of the CAT from

pSCS7 decreased to 15 and 10%, respectively, of the original activity under the same conditions. At 1.0 mM-HgCl₂, the CAT from pSCS6 retained 50% of its original activity while no enzyme activity could be detected for the CAT from pSCS7.

Discussion

Nucleotide sequences of seven staphylococcal *cat* genes, carried by the plasmids pC221 (Brenner & Shaw, 1985; Projan *et al.*, 1985), pC194 (Horinouchi & Weisblum, 1982), pUB112 (Brückner & Matzura, 1985), pSCS1 (Schwarz *et al.*, 1991), pSCS5 (Schwarz & Cardoso, 1991a), pSCS6 (Cardoso & Schwarz, 1992b) and pSCS7 (Schwarz & Cardoso, 1991b), have been used for phylogenetic or comparative studies. Six staphylococcal

Fig. 3. Agarose gel electrophoresis of the native CAT variants from pSCS6 (lane 1) and pSCS7 (lane 2).

Fig. 4. Influence of mercuric ions on CAT activity. O, Activity of the pSCS6-encoded CAT; Δ , activity of the pSCS7-encoded CAT. CAT activity is expressed as a percentage of the activity of the untreated control.

CAT enzyme variants have been distinguished. Four were designated A, B, C and D (Fitton & Shaw, 1979). However, no genetic analysis has been conducted for the CAT variants A, B and D (Shaw, 1983). Although the CAT encoded by the *S. aureus* plasmid pC194 has not been isolated and characterized biochemically, it was considered to be a fifth staphylococcal CAT variant based on data predicted from its nucleotide sequence (Shaw, 1983). The CAT encoded by the *S. haemolyticus*

plasmid pSCS5 represented the sixth staphylococcal CAT variant (Schwarz & Cardoso, 1991a). Thus, comparative data on the deduced amino acid sequences and the biochemical properties of the CAT proteins are available only for the CAT C variant encoded by the *S. aureus* plasmid pC221 (Fitton & Shaw, 1979; Brenner & Shaw, 1985) and the pSCS5-encoded CAT variant from *S. haemolyticus* (Schwarz & Cardoso, 1991a). The biochemical characteristics of the pSCS6- and pSCS7-encoded CAT variants presented in this study provide an opportunity to correlate properties of those proteins with the deduced amino acid sequences.

The pSCS6-encoded CAT variant corresponded closely to the pC221-encoded CAT C on the basis of their predicted amino acid sequences (Cardoso & Schwarz, 1992b). Only four amino acids of the pSCS6-encoded CAT monomer differed from the CAT C sequence. These amino acid exchanges occurred at positions which were not important for the structure and the function of the enzyme. The pSCS6- and pC221-specified CATs exhibited highest activities at pH 8.0; their K_m values for chloramphenicol were 2.5 μM and their K_m values for acetyl-CoA varied only slightly, being 59 μM for the CAT from pSCS6 and 61 μM for the CAT from pC221. Moreover, both enzymes were thermostable and were relatively insensitive to mercuric ions (Fitton & Shaw, 1979; Shaw, 1983; Shaw *et al.*, 1970). Unfortunately, the isoelectric point of the pC221-encoded CAT variant is not known. The isoelectric point of the pSCS6-encoded CAT was determined here to be pH 5.7, differing substantially from the values for the CAT variants encoded by the plasmids pSCS5 (pH 6.15) (Schwarz & Cardoso, 1991a) and pSCS7 (pH 5.2).

The CAT variants from pSCS5 and pSCS7 were found to be related on the basis of their predicted amino acid sequences (Schwarz & Cardoso, 1991b). However, the carboxy-terminal amino acid sequences differed completely. Nevertheless, these two CAT variants were assigned to the same evolutionary group, which is different from those represented by the pC221- and pC194-encoded CAT variants (Schwarz & Cardoso, 1991b). The relationship between the CATs from pSCS5 and pSCS7 was also suggested by the observation that both CAT variants were very susceptible to mercuric ions. This property has also been described for the CAT B variant from *S. epidermidis* (Shaw *et al.*, 1970). Moreover, the CAT from pSCS7 and CAT B corresponded closely in their K_m values. Both enzymes exhibited K_m values for chloramphenicol of 2.7 μM and for acetyl-CoA of approximately 56 μM . The other HgCl₂-sensitive CAT variant, from pSCS5, differed only slightly in its K_m values from these two CAT variants. The CAT from pSCS7 and CAT B also demonstrated highest activity at pH 8.0, while the pH optimum for

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CAT pSCS6  MTFNIIKLENWDRKEYFEHYFNQQTYSITKEIDITLFKDMSSKKKGYEIY  50
           * * * * *
CAT pSCS7  MTFNIIINLETWDRKEYFNHYFNQQTYSVTKELDITLLKSMIKNKGYELY  50

CAT pSCS6  PSLIYAIMEVVNKNKVFRGTGINSNKLGYWDKLNPLYTVFNKQTEKFTNI 100
           * * * * *
CAT pSCS7  PALIHAIVSVINRNKVFRGTGINSNKLGYWDKLEPLYTVFNKETENFSNI 100

CAT pSCS6  WTESDNNFTSFYNNYKNDLLEYKDKEEMFPKKPIPENTLPISMIPWIDFS 150
           *** * * * *
CAT pSCS7  WTESNASFTLFYNSYKNDLIKYKDKNEMFPKKPIPENTVPISMIPWIDFS 150

CAT pSCS6  SFNLNIGNNSNFFLLPIITIGKFYSENNKIYIPVALQLHHAUCDGYHASLF 200
           * * * * *
CAT pSCS7  SFNLNIGNNSRFLPIITIGKFYSKDDKIYLPFPLQVHHAUCDGYHVSLE 200

CAT pSCS6  INEFQDIKKVDDWI 215
           * * *
CAT pSCS7  MNEFQNIIR 209

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Fig. 5. Comparison of the predicted amino acid sequences of the CAT variants encoded by the plasmids pSCS6 (Cardoso & Schwarz, 1992b) and pSCS7 (Schwarz & Cardoso, 1991b). The amino acid sequences are displayed in the single-letter code; differences between the two CAT amino acid sequences are indicated by asterisks.

CAT from pSCS5 was slightly lower, at pH 7.8 (Schwarz & Cardoso, 1991a). The previously reported staphylococcal CAT variants B, C and D as well as the CAT variants encoded by the plasmids pSCS5, pSCS6 and pSCS7 were thermostable. Only CAT variant A was found to be thermolabile (Shaw, 1975).

All staphylococcal CAT variants exhibited the same quaternary structure. The M_r value of the pSCS6- and pSCS7-encoded CAT monomers was determined by SDS-PAGE to be 23000, a result in good accordance with the M_r values calculated from the predicted pSCS6- and pSCS7-encoded CAT amino acid sequences. Moreover, these M_r values corresponded very well to the value of 22500, previously published for the monomers of the staphylococcal CAT variants A, B, C and D (Zaidenzaig *et al.*, 1979), and that of 23000, determined for the pSCS5-encoded CAT monomer (Schwarz & Cardoso, 1991a). The experimentally determined M_r values of 70000 for the native CAT variants from pSCS6 and pSCS7 suggested that they exist as trimers composed of three identical subunits. A trimeric structure has previously been shown by X-ray crystallography of the R-factor-encoded type III CAT from *Escherichia coli* (Leslie *et al.*, 1988).

The data presented in this study demonstrate that despite the differences in their primary structure, the CAT variants encoded by the plasmids pSCS6 and pSCS7 share several properties, namely a trimeric quaternary structure, similar M_r values of the CAT monomers, a common pH optimum and similar thermostability. These two CAT enzymes varied slightly in their kinetic parameters. However, as a consequence of the

differences in their amino acid compositions they differed distinctly in their isoelectric points and their net charges. The active-site histidine at position 189 has previously been demonstrated to act as a general base catalyst in the acetylation of chloramphenicol (Leslie, 1990). This histidine residue was found to be conserved among all staphylococcal CAT variants so far investigated (Schwarz & Cardoso, 1991b), including those from pSCS6 and pSCS7 (Fig. 5). The high affinity of mercuric ions for histidine might explain the general sensitivity of these enzymes to mercuric ions. The enhanced sensitivity of CAT B and the CAT variants encoded by pSCS5 and pSCS7 to mercuric ions could be due to differences in the accessibility and reactivity of His-189 in these CAT variants.

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