A SCREENING METHOD FOR CELL WALL INHIBITORS USING A D-CYCLOSERINE HYPERSENSITIVE MUTANT

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Large numbers of antibiotics have been discovered in the past 40 years and many methods have been employed for discovery of novel antibiotics. One of them is the use of mutants that are highly sensitive to antibiotics. Recently β-lactam antibiotic-hypersensitive mutants of *Escherichia coli*[1] and *Pseudomonas aeruginosa*[2] and an aminoglycoside-hypersensitive mutant of *Klebsiella pneumoniae*[3] have been used in screening for new antibiotics.

D-Cycloserine (DCS) has a broad antibacterial spectrum[4] and acts on alanine racemase (EC 5.1.1.1) and D-alanine: D-alanine ligase (EC 6.3.2.4), which are involved in synthesis of the peptidoglycan layer of the cell wall in bacteria[5,6]. The antibiotics MK641/MK642[7,8] and alaphosphin[9] also have a similar mechanism of action. Good synergy in bactericidal activity was demonstrated in the combinations between these antibiotics and β-lactam antibiotics[10]. Therefore, DCS-type antibiotics are expected to be useful for clinical purposes, especially against β-lactam resistant bacteria. It is anticipated that a DCS-sensitive mutant should be useful in detecting minor and novel DCS-type antibiotics produced in fermentation broths. Based on this concept, we obtained a very sensitive and highly specific mutant to DCS derived from *Staphylococcus aureus* Newman.

In this paper we report the isolation and characterization of the DCS hypersensitive mutant C-33, and its use in screening for antibiotics.

Induction and isolation of mutants were achieved by treating *S. aureus* Newman cells with 100 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in 0.05 M Tris-malate buffer (pH 6.0) at 37°C for 15 minutes and then selecting in replicate nutrient agar (NA) plates with and without 1 μg/ml of DCS. The colonies which did not grow on plates containing DCS were selected from a plate without DCS. Of the many DCS-sensitive mutants cloned, strain C-33 was selected because of its high sensitivity and good growth at 37°C. The mutant C-33 showed similar growth to that of the parent one at 28°C and 42°C (data not shown). Therefore, *S. aureus* C-33 is not a ts-mutant.

The MIC of DCS against the mutant C-33 was 6 ng/ml, indicating that it was 8,000-fold more sensitive than the parent strain (MIC 50 μg/ml) to DCS. The inhibitory zones of DCS and other antibiotics against the C-33 and parent strains were determined by a conventional paper-disc method using NA medium at 37°C (Table 1). The mutant C-33 showed specific sensitivity to DCS and was sensitive to 0.016 μg/ml of DCS, while the parent strain showed no inhibitory zone even at 1 μg/ml. The C-33 and parent cells were equally sensitive to all other antibiotics and agents tested except Triton X-100.

To determine whether the antibiotics selected in the primary screen test by paper-disc assay were inhibitors of cell wall synthesis, the rates of incorporation of radioactive precursors into the acid-insoluble macromolecular fraction in *S. aureus* C-33 at various concentrations of DCS were measured as described previously[11] (Table 2). The syntheses of DNA, RNA and protein, assayed by measuring the incorporation of [^{3}H]-thymidine, [^{3}H]uridine and [^{3}H]leucine, respectively, were scarcely affected by incubation for 30 minutes with DCS, even at a concentration of 1 μg/ml which induced a relatively large inhibitory zone. However at the low concentration of 0.01 μg/ml, DCS prevented the incorporation of *N*-[^{14}C]acetylglucosamine, a precursor of cell walls. Antibiotics other than cell wall inhibitors had no effect on the incorporation of *N*-[^{14}C]acetylglucosamine in either *S. aureus* C-33 or *S. aureus* Newman (data not shown). Triton X-100, to which the mutant C-33 was more sensitive than the parent strain, also did not affect the incorporation of *N*-[^{14}C]-acetylglucosamine into C-33. These results showed that assay of incorporation of this radioactive precursor could also be used in the second test to detect inhibitors of cell wall synthesis.

The rates of incorporation of several radioactive precursors in the mutant C-33 and the parent strain were compared (Fig. 1). Growing

Table 1. Paper-disc assay using D-cycloserine (DCS)-hypersensitive mutant and its parent strain.

Antibiotic	Concentration (μg/ml)	Zone size (mm)	
		Parent	C-33
DCS	1	—	28.2
	0.25	—	22.9
	0.063	—	16.7
	0.016	—	9.9
Fosfomycin	10	18.2	18.2
Moenomycin	10	13.5	15.7
Vancomycin	100	14.7	15.3
Bacitracin*		10.4	10.1
Benzylpenicillin	0.5	20.0	19.0
Cephalexin	10	16.3	15.8
Gentiana violet	25	14.4	15.4
Albocycline	100	21.5	21.3
Glutamycin	100	15.0	14.3
Mitomycin C	4	15.5	15.7
Rifampicin	1	18.5	19.4
Erythromycin*		22.4	24.2
Kitasamycin*		21.6	21.5
Novobiocin*		27.2	26.3
Mikamycin*		24.0	24.6
Lincomycin*		25.4	25.7
Chloramphenicol*		27.8	29.3
Tetracycline*		27.6	30.2
Kanamycin*		21.6	22.0
Streptomycin*		19.6	20.3
Nalidixic acid*		19.0	21.2
Furazolidon*		20.9	21.0
Colistin*		—	—
Polymyxin B*		—	—
SDC	5,000	13.2	12.9
SDS	5,000	14.3	14.8
Triton X-100	10,000	—	13.1

Paper discs (6.5 mm in diameter) were used.
* Commercial discs from Showa Yakuhin Kako Co., Ltd.
SDC: Sodium dodecyl chloride.
—: No inhibitory zone was detected.

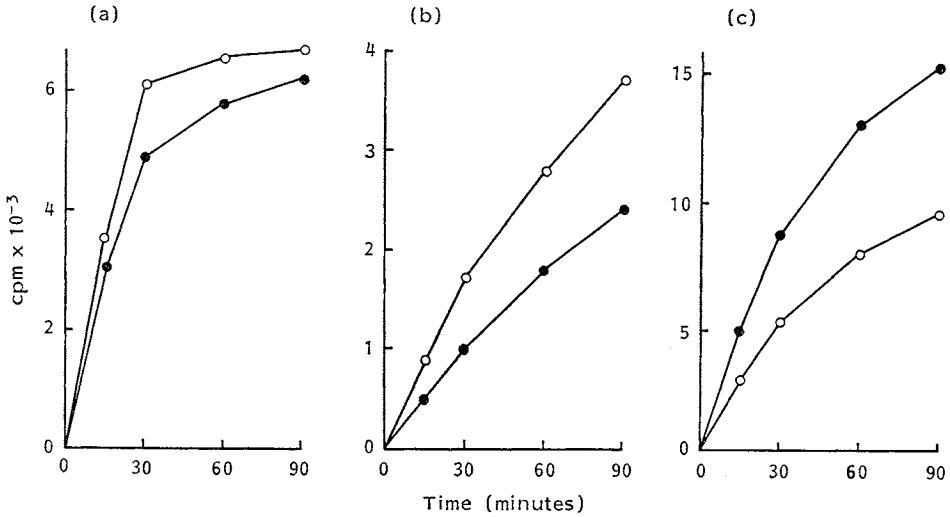
Table 2. Effect of D-cycloserine (DCS) on the incorporation of radioactive precursors into the acid-insoluble macromolecular fraction in *Staphylococcus aureus* C-33.

DCS conc (μg/ml)	Incorporation (%)			
	[^{14}C]GlcNAc	[^{3}H]Thd	[^{3}H]Urd	[^{3}H]Leu
0	100	100	100	100
0.01	63	106	105	102
0.1	39	105	102	100
1	19	95	94	96

Abbreviations: [^{14}C]GlcNAc, *N*-[^{14}C]Acetylglucosamine; [^{3}H]Thd, [^{3}H]thymidine; [^{3}H]Urd, [^{3}H]-uridine; [^{3}H]Leu, [^{3}H]leucine.

Fig. 1. Incorporations of *N*-[^{14}C]acetylglucosamine (a), L-[^{14}C]alanine (b), and D-[^{14}C]alanine (c) into the acid-insoluble macromolecular fraction of growing cells of *Staphylococcus aureus* Newman and C-33.

○ *S. aureus* Newman, ● *S. aureus* C-33.



cells of *S. aureus* C-33 showed higher incorporation of D-[^{14}C]alanine into the acid-insoluble macromolecular fraction than *S. aureus* Newman cells while showed less incorporation of *N*-[^{14}C]-acetylglucosamine and L-[^{14}C]alanine. This seems to show that one of the mechanisms of sensitivity of the mutant to DCS could be a change in the D-alanine transport system.

By screening about 8,000 soil isolates, including bacteria, fungi and actinomycetes, one antibiotic, TA-243[12], was discovered, and three known antibiotics, DCS[4], *O*-carbamyl-D-serine[13] and FR-900148[14], were identified.

DCS was isolated from cultures of a wide variety of soil isolates, of which *Streptomyces* was the commonest, accounting for 0.5% of the total. DCS was also isolated from cultures of three rare actinomycetes and one *Pseudomonas* sp.

TA-243 produced by *Streptomyces griseofuscus* OFR 1388, a new antibiotic discovered by this screening procedure, is a novel inhibitor of cell wall synthesis. TA-243 was actively transported into bacterial cells by peptide transport system and was subsequently hydrolyzed by intracellular aminopeptidases to yield aminooxysuccinic acid. Its primary intracellular target site was alanine racemase (manuscript in preparation).

The strain C-33 is the first example of DCS-hypersensitive mutants. The above results indicate that it is a useful tool in screening work for new cell wall inhibitors.

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