STUDIES ON THE ACTION OF O-CARBAMYL-D-SERINE IN *BACILLUS SUBTILIS*

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(1) D-Alanyl-D-alanine, D-alanine, L-alanine, D, L-α-aminobutyric acid, D-serine, and L-methionine were found to reverse the action of O-carbamyl-D-serine on *Bacillus subtilis* in synthetic medium. (2) When O-carbamyl-D-serine was added to the growth medium a relative increase of L-alanine and ammonia, and an accumulation of a nucleotide peptide were observed. The structure of the nucleotide peptide was identified as UDP-MurNAc-Ala-Glu-DAP. (3) Several compounds related to O-carbamyl-D-serine were synthesized and tested for their antimicrobial activity. Only O-carbamyl-D-serine methyl ester exhibited activity, which could be reversed by D-alanyl-D-alanine, D-alanine and L-alanine. N-D-Alanyl-O-carbamyl-D-serine which has been reported to be enzymatically synthesized by D-alanyl-D-alanine synthetase of *Streptococcus faecalis* R, did not have antimicrobial activity. (4) We concluded that the primary site of the action of O-carbamyl-D-serine in *Bacillus subtilis* is an enzymatic system controlling racemization of alanine. A similar conclusion was previously reached by LYNCH and NEUHAUS by use of enzymatic methods in *Streptococcus faecalis* R.

O-Carbamyl-D-serine is an antibiotic which is known to be an inhibitor of cell wall biosynthesis. TANAKA *et al.*[1,2] reported that the antibiotic activity was reversed by D-alanine. Incorporation of C^{14}-D,L-glutamic acid into cell wall fractions was inhibited and in addition there was an intracellular accumulation of N-acylamino sugar in the presence of the antibiotic. Furthermore, a potential synergistic action with D-cycloserine was also reported[3].

Recently, the biosynthetic pathway of glycopeptide, which is considered to be a common basal structure of bacterial cell walls, was elucidated at the enzymatic level. In 1966, LYNCH and NEUHAUS[4] reported their experiments indicating that the primary site of action of O-carbamyl-D-serine is alanine racemase. This conclusion was based on the following observations in *Streptococcus faecalis* R: (i) accumulation of UDP-MurNAc-L-Ala-D-Glu-L-Lys; (ii) absence of D-alanyl-O-carbamyl-D-serine accumulation in bacterial cultures grown in the presence of O-carbamyl-D-serine; and (iii) effective inhibition of alanine racemase.

We studied, in *Bacillus subtilis*, the reversing action of amino acids and a peptide (D-Ala-D-Ala) on the antibiotic activity by a semi-quantitative bioassay method. In addition, an analysis and comparison was made of acid-soluble intracellular substances extracted from cells grown in the presence and absence of O-carbamyl-D-serine. Also studied was the antimicrobial activity of several synthetic compounds which are structurally related to O-carbamyl-D-serine. All the results obtained provided further support for the conclusion proposed by LYNCH and NEUHAUS.

Materials and Method

O-Carbamyl-D-serine (O-CS) used in these experiments was a by-product[5] of D-cycloserine fermentation in a pilot plant of our company, and was recrystallized twice before use; m.p., darkened above *ca.* 190°C and decomposed at 206~209°C, $[\alpha]_D^{25}$ 3.2°±2° (c 1.139, H_2O).

Anal. Calcd. for $C_4H_8O_4N_2$: C 32.43, H 5.44, N 18.91
Found: C 32.51, H 5.50, N 18.69

D- and L-Alanine were purchased from Sigma Chemical. They were recrystallized and their optical purity confirmed; D-Alanine: $[\alpha]_D^{24.5}$ −13.8°±0.5° (c 4.165, 6 N HCl), L-Alanine: $[\alpha]_D^{22}$ +13.9°±1° (c 2.076, N HCl).

D-Serine was prepared from O-CS by acid hydrolysis: $[\alpha]_D^{22}$ +7.5°±2° (c 1.034, H_2O).

O-Benzyl-D-serine was synthesized by Dr. K. INOUE of our laboratory: $[\alpha]_D^{24.5}$ −7.2°±0.3° (c 2.046, N HCl).

The medium for *Bacillus subtilis* PCI 219 was a glutamate-glucose-mineral medium[3], which consisted of 0.1 % KH_2PO_4, 0.07 % $MgSO_4 \cdot 7H_2O$, 0.1 % NaCl, 0.003 % $FeSO_4 \cdot 7H_2O$, 0.2 % $(NH_4)_2HPO_4$, 0.2 % L-sodium glutamate and 0.5 % glucose (pH 7.0).

Observation of reversing action: The common cylinder agar method was employed. Each amino acid or peptide in several concentrations was added to the medium inoculated with *B. subtilis* spores at a concentration of 5×10^6 cells/ml. The spores used were previously washed ten times with distilled water. Solutions of O-CS were placed in the cylinder and after incubation the diameters of the inhibitory zones were measured.

Preparation of acid-soluble extract of *B. subtilis*: A spore suspension of *B. subtilis* PCI 219 was inoculated in a glutamate-glucose-mineral medium at a concentration of 10^7 cells/ml and shaken for about 20 hours at 27°C. A 40-ml portion of the fully grown culture (turbidity, as optical density at 650 mμ, *ca.* 1.35) was then inoculated into 400 ml of the medium and it was shaken for 4 hours at 27°C to half maximum growth (O.D. at 650 mμ, *ca.* 0.55). The cells were then collected by centrifugation and resuspended in the same volume of the medium containing O-CS at a concentration of 2 mM. The same inoculum was also added to a medium without O-CS as a control. After shaking for 90 minutes at 27°C, the cells were again collected, washed with distilled water twice and suspended in 5 ml of water. The cell suspension was heated in boiling water for 10 minutes to destroy all enzymatic activities. After cooling with ice, two extractions with 5 % trichloroacetic acid were carried out. The two extracts were combined, trichloroacetic acid removed by extraction with ether, and the remaining solution adjusted to pH 5.0 and lyophilized.

Paper chromatography: Two-dimensional paper chromatography on Toyo roshi No. 51 with 95 % ethanol-1 M ammonium acetate, pH 7.2 (7.5 : 3) and isobutyric acid-0.5 M NH_4OH (5 : 3)[6] was employed for the cell extracts. Nucleotides were located on the print made by a letter copying machine (Quik Copy, made by Fuji Photo Film Co., Ltd.) by transmitted ultraviolet light as ultraviolet absorbing zones. For the isolation of the nucleotides, chromatography was carried out with each solvent preparatively.

Determination of nucleotide peptide: The UV-absorbing zones on the paper were extracted with water. The extracts were measured for UV-absorption in 0.01 N HCl and 0.01 N NaOH, and then hydrolyzed with constant boiling HCl at 105°C for 24 hours. The hydrolysates were analyzed by a Hitachi automatic amino acid analyzer. Muramic acid was also determined by the analyzer in direct comparison with an authentic sample. Phosphorous content was determined by MARINETTI's method[7].

Amino acid analysis: The cell extract was directly subjected to automatic amino acid analysis to determine amino acid or other ninhydrin-positive substances present. A 50-cm high column was used with 0.2 M citrate buffer, pH 3.25, and the buffer changed to 0.2 M, pH 4.25, and 0.7 M, pH 5.28, after 3 and 5 hours respectively, so as to cover a wide range of amino acids.

Gas-liquid chromatography: The configuration of alanine in the cell extract was determined by gas-liquid chromatography. A portion of the cell extract was treated with activated charcoal (Darco G-60) and charged on a Dowex 50×8 (H) column. Amino acids were eluted with 0.5 N NH_4OH and then converted to N(L-α-chloroisovaleryl)-amino acid methyl esters[8]. The resulting derivatives form the cell extract and authentic ones from D- and L-alanine were chromatographed at 81°C on column of EGSS-X, 1 % 1.5 m with a Shimazu Gas chromatograph GC-1B.

Synthetic Studies

O-Acetyl-D-serine hydrobromide: N-*t*-Butoxycarbonyl-O-benzyl-D-serine, synthesized by Dr. N. YOSHIDA of our laboratory, was dissolved in acetic acid containing 30 % hydrogen bromide and allowed to stand for 1 hour at room temperature. Dry ether was added to the mixture to yield a crystalline precipitate. Recrystallization from ethanol-ethyl acetate gave fine colorless plates (yield 82 %), m.p. 164~167°C (decomp.), $[\alpha]_D^{22.5}$ −11.5°±1° (c 2.045, H_2O).

Anal. Calcd. for $C_5H_9O_4N \cdot HBr$: C 26.33, H 4.39, N 6.16, Br 35.06
Found: C 26.10, H 4.67, N 6.41, Br 35.35

N-*t*-Butoxycarbonyl-O-carbamyl-D-serine: To a solution of O-CS (10 mmoles) and $NaHCO_3$ (24 mmoles) in 40 ml of water-dioxane (1 : 1), *t*-butyl azidoformate (12 mmoles) was added. The reaction was continued for 20 hours at 40°C with stirring. The reaction mixture was cooled, adjusted to pH 4.8 with HCl, and concentrated. Repeated extraction was carried out with ethyl acetate at pH 2.0. The extract was then dried and concentrated to dryness. Dry ether was added to the residue resulting in colorless plates, which were recrystallized from ethanol-water (yield 67 %), m.p. 140.5~142.0°C, $[\alpha]_D^{25}$ −14.4°±2° (c 1.031, CH_3OH).

Anal. Calcd. for $C_9H_{16}O_6N_2$: C 43.54, H 6.50, N 11.29
Found: C 43.62, H 6.51, N 11.34

O-Carbamyl-D-serine methyl ester: O-CS was dissolved in methanol saturated with HCl, and allowed to stand for 40 hours at room temperature. The solution was evaporated to dryness, and the residue was crystallized from ethanol to yield colorless needles (yield 46 %), m.p. 138~142°C (decomp.), $[\alpha]_D^{22}$ −10.5°±2° (c 1.031, H_2O).

Anal. Calcd. for $C_5H_{10}O_4N_2 \cdot HCl$: C 30.23, H 5.54, N 14.11, Cl 17.88
Found: C 30.16, H 5.87, N 14.24, Cl 18.22

N-D-Alanyl-O-carbamyl-D-serine*: To a solution of O-CS (2 mmoles) in water (10 ml), pyridine (20 ml) and a trace amount of acetic acid and N-benzyloxycarbonyl-D-alanine N-hydroxysuccinimide ester (2 mmoles) were added. The mixture was stirred for 5 hours at about 50°C, and then allowed to stand for 2 days at room temperature. This reaction proceeded at a slow rate, probably because O-CS was poorly soluble in the solvent. The reaction mixture was concentrated, and the residue (unreacted O-CS) was filtered off. The filtrate was evaporated to drynss, and dissolved in ethyl acetate. After transfer to 5 % $NaHCO_3$ and re-extraction with ethyl acetate at an acid pH, the ethyl acetate solution was dried and concentrated to a syrupy solution. By ether extraction, N-benzyloxycarbonyl-D-alanine was removed and N(N-benzyloxycarbonyl-D-alanyl)-O-carbamyl-D-serine remained as a colorless crystalline residue (yield 8 %). Subsequent hydrogenolysis in acetic acid in the presence of palladium black gave N-D-alanyl-O-carbamyl-D-serine in theoretical yield. Recrystallization from water-ethanol geve colorless needles which decomposed at above *ca.* 215°C, $[\alpha]_D^{24}$ −34.8°±2° (c 1.066, N AcOH).

Anal. Calcd. for $C_7H_{13}O_5N_3$: C 38.35, H 5.98, N 19.17
Found: C 37.95, H 5.88, N 19.45

* LYNCH and NEUHAUS[4] reported that this compound was synthesized by HERMAN PLAUT with the same procedure used for the synthesis of O-carbamyl-D-serine from N-benzyloxycarbonyl-D-alanyl-D-serine *p*-nitrobenzyl ester.

O-Carbamyl-D-seryl-D-alanine: N-*t*-Butoxycarbonyl-O-carbamyl-D-serine N-hydroxysuccinimide ester (2 mmoles) was dissolved in 20 ml of tetrahydrofuran, and mixed with an aqueous solution (10 ml) of D-alanine (2 mmoles) and $NaHCO_3$ (2 mmoles). The mixture stood for 72 hours at room temperature and was then concentrated at pH 2.0. The resulting oily precipitate was separated and extracted with chloroform. A slight excess of dicyclohexylamine was added to the extract, and it was washed with water, dried, and concentrated to dryness. The residue, after washing with hot ethyl acetate, was crystallized from methanol-ethyl acetate to give colorless needles of N-*t*-butoxycarbonyl-O-carbamyl-D-seryl-D-alanine dicyclohexylamine salt (yield 40 %), m. p. 146~148°C, $[\alpha]_D^{25}$ $-1.3° \pm 2°$ (c 1.085, CH_3OH).

Anal. Calcd. for $C_{24}H_{44}O_7N_4 \cdot {}^1/_2H_2O$: C 56.58, H 8.84, N 11.09
Found: C 56.47, H 9.19, N 11.09

After removing dicyclohexylamine by Dowex×50 (H^+), treatment with cold tetrahydrofuran saturated with hydrochloric acid gave an amorphous powder of O-carbamyl-D-seryl-D-alanine, m. p. 193~194°C.

Anal. Calcd. for $C_7H_{13}O_5N_3$: C 38.38, H 5.98, N 19.17
Found: C 38.31, H 5.75, N 18.74

D-Alanyl-D-alanine: N-Benzyloxycarbonyl-D-alanyl-D-alanine benzyl ester was synthesized from N-benzyloxycarbonyl-D-alanine and D-alanine benzyl ester by the mixed anhydride method[9] (yield 82 %) and the carbodiimide method[10] (yield 94 %). By hydrogenolysis with palladium black in acetic acid, D-alanyl-D-alanine was obtained as colorless needles crystallized from water-ethanol and they decomposed at above *ca.* 250°C, $[\alpha]_D^{23}$ $+27.7° \pm 1°$ (c 2.050, N AcOH).

Anal. Calcd. for $C_6H_{12}O_3N_2$: C 44.99, H 7.55, N 17.49
Found: C 44.56, H 7.80, N 17.48

Results

Reversing action of amino acids and a peptide:

Reversing action of several amino acids and one peptide on the antibiotic activity of O-CS was measured by the standard cylinder agar method. The results obtained are listed in Table 1. D-Alanyl-D-alanine reversed the activity of O-CS at a concentration of 0.031 mM, and D-alanine at 0.25 mM. The ratio of the concentrations was about 1 : 8, although the ratio of the strength of the reversing actions of the two compounds should be considered as 4 : 1, since 1 mole of D-alanyl-D-alanine corresponds to 2 moles of D-alanine as material for the biosynthesis of cell walls. In the case of L-alanine, reversing action was observed partially at 0.5 mM and completely at 1.0 mM. Reversal was also observed with D,L-α-aminobutyric acid and D-serine, and a weak reversing action was observed with L-methionine.

Isolation and determination of a nucleotide peptide:

The cell extract prepared from the O-CS containing medium was chromatographed in two dimensions. A main UV-absorbing zone was found near the origin. Mobility of the UV-absorbing substance was somewhat slower than that of adenosine triphosphate which was used as a reference compound in both solvent systems. To determine the amount of the substance, an aliquot of the cell extract corresponding to 40 ml of the culture was applied and then the optical density at 262 mμ measured after extraction with water. The molar ratio of the substance to the glutamic acid content in the cell extract was estimated to be 0.5. In the case of the O-CS-free medium, no UV-absorbing

zone was found at the same position even when a considerable amount of the cell extract was applied.

This main UV-absorbing substance was ninhydrin-positive, and showed a UV-absorption maximum at 262 mμ in both 0.01 N HCl and 0.01 N NaOH. The spectra agreed with that of uridylic acid. The analysis of a hydrolysate of the substance gave the constituents listed in Table 2.

Although the sequence has not been completely determined, it is reasonable to predict from its composition that this substance is the nucleotide tripeptide, UDP-MurNAc-Ala-Glu-DAP[12].

In a preparative scale paper chromatographic separation, three additional UV-absorbing substances were found. They showed higher mobilities on the chromatogram than did the main substance described above. After extraction from the chromatogram, these materials showed the following UV maxima: in 0.01 N HCl, 257, 258 and 281 mμ, in 0.01 N NaOH, 258, 260 mμ, and no measurement. Acid hydrolysates of these substances gave no significant amount of amino acids.

Table 1. Reverse action of amino acids and a peptide to the antibiotic activity of O-carbamyl-D-serine

Amino acid or peptide in the medium		O-Carbamyl-D-serine in the cylinder and zone of inhibition in mm	
		10 mM	2 mM
None		32.5	20.5
L-Alanine	2.0 mM	0	0
	1.0 //	29.0	0
	0.5 //	32.0	19.0
D-Alanine	0.25 //	0	0
	0.125 //	31.0	20.5
D-Alanyl-D-alanine	0.062 //	0	0
	0.031 //	27.0	0
	0.016 //	32.0	16.5
	0.008 //	31.5	19.5
D,L-α-Aminobutyric acid	0.5 //	13.0	0
	0.25 //	26.0	12.0
	0.125 //	30.5	18.0
L-Serine*	1.0 //	—	—
D-Serine**	0.25 //	19.0	0
	0.125 //	25.5	11.0
	0.062 //	32.0	18.0
Glycine	1.0 //	33.0	20.0
L-Valine	1.0 //	34.0	22.0
L-Phenylalanine	1.0 //	34.0	22.0
L-Threonine	1.0 //	33.5	20.5
L-Aspartic acid	1.0 //	32.5	20.0
L-Ornitin	1.0 //	32.5	21.5
L-Lysine	1.0 //	32.5	21.0
L-Cystine	1.0 //	32.0	19.0
L-Methionine	1.0 //	27.0	13.0

* L-Serine exhibited an antimicrobial activity at 0.25 mM in this medium and below this concentration, no reversing action was observed.

** D-Serine exhibited an antimicrobial activity at 4.0 mM, and below this concentration, a reversing action was observed.

Analysis of cell extracts:

When the acid-soluble cell extract from the O-CS-containing medium was analyzed by the automatic amino acid analyzer, peaks for the nucleotide peptide, O-CS, glutamic acid, alanine, ammonia and an unknown compound were found as shown in Fig. 1. In the case of the extract from the O-CS-free medium, on the other hand, no peaks of nucleotide peptide or O-CS were found.

The molar ratios to glutamic acid are shown in Table 3. Sodium glutamate was present in both media (O-CS present and absent). Therefore, the glutamic acid content in both cell extracts was considered to be comparable. In this table some deviation was shown in the two experiments, probably because of the difficulty in controlling culture age and cell washing exactly in each experiment. It is, however, certain that the relative amounts of alanine and ammonia to glutamic acid increase significantly when

Table 2. Composition of the nucleotide peptide

Component	Found molar ratio*
Uridine	1.00
Glutamic acid	0.74
Alanine	0.64
α,ε-Diaminopimelic acid	1.03
Muramic acid	0.43
Phosphorous	1.84

* Expressed in molar ratio to uridine content determined by UV-absorption. Phosphorous content was expressed as gram atom.

Fig. 1. Amino acid analysis on acid-soluble cell extracts

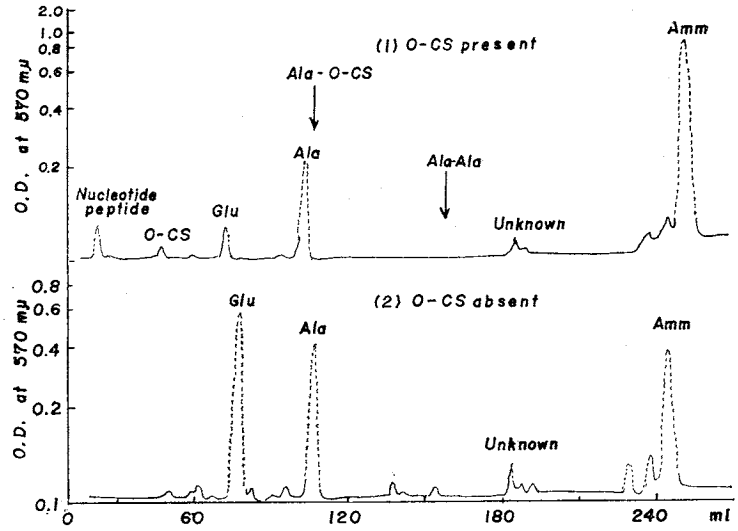

Table 3. Analyses on the acid-soluble extracts from *B. subtilis* grown in the presence or absence of O-CS

Compound	Relative amount in molar ratio			
	O-CS present		O-CS absent	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Glutamic acid	1.00	1.00	1.00	1.00
L-Alanine	1.73	4.30	0.41	0.75
Ammonia	5.04	13.1	0.93	0.43
O-CS	+	0.25	—	—
Nucleotide peptide	0.50*	+	0	0

* Determined by UV-absorption after isolation by paper chromatography.

Fig. 2. Scheme of biosynthetic pathway of glycopeptide

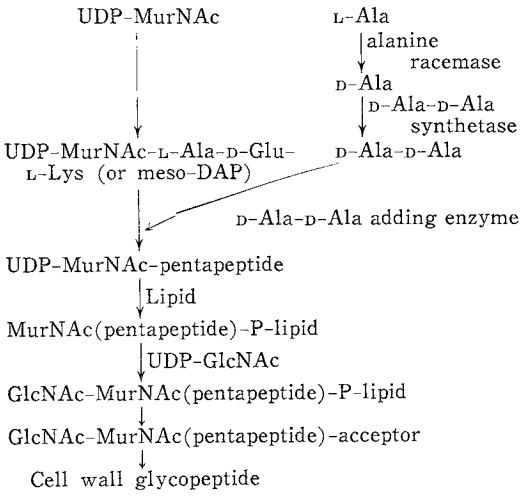

Table 4. Antimicrobial activity of compounds related to O-carbamyl-D-serine to *B. subtilis* in synthetic medium

Compound in the cylinder	Concentration (mM)	Inhibitory zone (mm)
O-Carbamyl-D-serine (as reference)	10	30.0
	2	18.0
O-Acetyl-D-serine	10	0
	2	0
O-Benzyl-D-serine	10	0
	2	0
N-*t*-Butoxycarbonyl-O-carbamyl-D-serine	10	0
	2	0
O-Carbamyl-D-serine methyl ester	10	21.0
	2	13.0
N-D-Alanyl-O-carbamyl-D-serine	10	0
	2	0
O-Carbamyl-D-seryl-D-alanine	10	0
	2	0

Table 5. Reversing action of L-alanine, D-alanine and D-alanyl-D-alanine on the antimicrobial activity of O-carbamyl-D-serine methyl ester

Compound	Concentration (mM)	O-Carbamyl-D-serine methyl ester	
		10 mM	2 mM
None	—	18.0	13.0
L-Alanine	0.25	0	0
	0.125	14.5	0
D-Alanine	0.031	0	0
	0.016	14.0	0
D-Alanyl-D-alanine	0.008	0	0
	0.004	12.0	0

Conditions and expressions were described in Table 1.

O-CS is present in the medium.

The configuration of alanine contained in both cell extracts was determined to be the L-form by the gas-liquid chromatographic technique. The N(L-α-chloroisovaleryl)-alanine methyl esters derived from both extracts and authentic L-alanine showed the same retention time (3.40 minutes) under the experimental conditions described.

Antimicrobial activity of the compounds related to O-carbamyl-D-serine:

Six compounds prepared in this work were investigated for their antimicrobial activity against *B. subtilis* in the synthetic medium by means of the cylinder agar method. As shown in Table 4, only O-carbamyl-D-serine methyl ester exhibited activity. Its activity was completely reversed by L-, and D-alanine and D-alanyl-D-alanine (Table 5).

Discussion

The general pattern of the synthetic pathway of glycopeptide is now believed to be that described in Fig. 2. L-Alanine, D-glutamic acid and L-lysine (or meso-DAP) are added sequentially to UDP-MurNAc, and catalyzed by soluble enzymes[6] to form UDP-MurNAc-tripeptide. L-Alanine is converted to D-alanine by the catalysis of alanine racemase, after which D-alanyl-D-alanine is synthesized and added on to the UDP-MurNAc-tripeptide. This reaction is catalyzed by D-alanyl-D-alanine synthetase and D-alanyl-D-alanine adding enzyme[11,12]. By the aid of particulate enzyme fractions containing lipid[13,14], the UDP-MurNAc-pentapeptide is bound with GlcNAc, and the hetero-disaccharide polymerized through a lipid intermediate [described as GlcNAc-MurNAc-(pentapeptide)-acceptor in Fig. 2]. Then the linear polymer is cross-linked by transpeptidase and D-alanine carboxypeptidase[15,16].

The enzymatic reactions cited in this scheme have been reported in strains of *Staphylococcus*, *Streptococcus*, *Micrococcus*, and *Escherichia coli*, but not in *Bacillus subtilis*. However, it is unlikely that the *B. subtilis* pathway of glycopeptide synthesis differs substantially from this scheme.

An accumulation of UDP-MurNAc-Ala-Glu-DAP in log-phase cell of *B. subtilis* by O-CS was observed in this experiment. This indicated that the site of action of the antibiotic was not before UDP-MurNAc-tripeptide. This is also responsible for the N-acylamino sugar accumulation observed by TANAKA *et al.*[1,2] and a similar accumulation of UDP-MurNAc-L-Ala-D-Glu-L-Lys in *Streptococcus faecalis* R observed by LYNCH and NEUHAUS[4], which was determined by enzymatic methods. No accumulation of UDP-MurNAc-pentapeptide was observed here and may suggest that O-CS did not act on the lipid cycle. Therefore, the site would be present at the area involving synthesis of UDP-MurNAc-pentapeptide from UDP-MurNAc-tripeptide, which includes the enzymatic reactions by alanine racemase, D-Ala-D-Ala synthetase and D-Ala-D-Ala adding enzyme.

The reversing action of D-Ala-D-Ala was most effective, and that of D-alanine and L-alanine followed this; the strength of the reverse actions of D-alanine and L-alanine were about 1/4 and 1/8, respectively, of the strength of the reversing action of D-Ala-D-Ala with respect to their molecular size. This suggests that the site of the action of O-CS is more likely at the point of alanine racemization or the D-Ala-D-Ala synthesis rather than at the addition reaction point of D-Ala-D-Ala. Furthermore, a relative increase in L-alanine was observed in the cell of *B. subtilis* grown in the presence of O-CS, indicating that the most probable site is that involving racemization of alanine. Even in the normal culture, the occurrence of L-alanine was observed, but not that of D-alanine. It might be proposed that the steric conversion of L-alanine to D-alanine plays an important role which controls the velocity of the series of enzymatic reactions. In the cell free system

of *Streptococcus faecalis* R, a competitive inhibition of O-CS for alanine racemase, but not for the D-Ala-D-Ala synthetase and the adding enzyme, was reported[4].

The relative increase of intracellular content of ammonia by O-CS is reasonable if the route after L-alanine, which can be synthesized by a reversible enzymatic reaction from pyruvate and pooled ammonia[17], is blocked. The reverse action of D,L-α-amino-n-butyric acid and D-serine was also recognized in *Streptococcus faecalis* R[4].

LYNCH and NEUHAUS[4] reported that when D-Ala-D-Ala synthetase was incubated with O-CS and D-alanine, D-alanyl-O-carbamyl-D-serine (D-Ala-O-CS) was formed together with D-Ala-D-Ala, and this unusual compound was then incorporated into the nucleotide pentapeptide by D-Ala-D-Ala adding enzyme. They also reported no accumulation of D-Ala-O-CS in the intact cell system, but the experimental data was not published. The present experiment with *B. subtilis* provides the evidence for lack of accumulation of D-Ala-O-CS by means of amino acid analysis. However, it is possible that D-Ala-O-CS formed in the intact cell is immediately incorporated into the nucleotide pentapeptide which then results in the formation of a defective cell wall structure. However, this is considered to be unlikely in view of the fact that D-Ala-O-CS had no antimicrobial activity.

Among six compounds tested for antimicrobial activity, only O-carbamyl-D-serine methyl ester was active. The activity was reversed by a series of compounds: L-alanine, D-alanine and D-Ala-D-Ala. From these results it is suggested that the ester may act like O-CS itself.

Addendum

The following experiment was carried out according to a referees' advice. O-Carbomyl-D-serine methyl ester was added to a log-phase culture of *B. subtilis* and incubated for 4 hours at 37°C. The ester was measured by the ferric hydroxamate method described by M. EBATA[18]. As a result, no hydroylsis of the ester was found during the incubation. This indicated that the ester was quite resistant to any esterases of the organism and that the ester itself exhibited the antimicrobial activity described in the text.

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