CEPHABACINS, NEW CEPHEM ANTIBIOTICS OF BACTERIAL ORIGIN

I. DISCOVERY AND TAXONOMY OF THE PRODUCING ORGANISMS AND FERMENTATION

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Three Gram-negative bacteria produce new cephem antibiotics, named cephabacins, with unique 3-side chains. Cephabacins include F group antibiotics with a 7-formylamino substituent and H group antibiotics without the substituent. The producing bacteria were taxonomically characterized and designated as *Lysobacter lactamgenus* sp. nov. YK-90 and *Xanthomonas lactamgena* sp. nov. YK-278 and YK-280.

In the course of our screening program aimed at detecting new β-lactam antibiotics, several strains of Gram-negative bacteria isolated from soil and plant samples produced new types of cephem antibiotics named cephabacins. A series of cephabacin compounds were designated as cephabacin $F_{1\sim9}$ and cephabacin $H_{1\sim6}$ according to the presence or absence of a formylamino substituent at the 7-position and of a guanidyl group in the 3-side chains, and the difference in the number of L-alanine or L-serine residues in the 3-side chains[1,2]. Cephabacin F group antibiotics with the 7-formylamino substituent were highly resistant to hydrolysis by various types of β-lactamases[3].

Cephalosporin antibiotics have been believed to be exclusively fungal metabolites. However, the discovery of cephamycin C produced by *Streptomyces*[4,5] opened up a new era of screening for β-lactam antibiotics from natural sources. In 1982, deacetoxycephalosporin C was detected in cell extracts of Gram-negative bacteria, *Flavobacterium* and *Xanthomonas* species[6]. The same strain of *Flavobacterium* has recently been shown to also produce two 7-formamidocephalosporins in its culture filtrate[7].

The discovery and taxonomy of the producing organisms of cephabacins and their fermentation are described.

Discovery of the Producing Organisms

The presence of β-lactam antibiotics in culture filtrates of strains YK-90, YK-278 and YK-280 grown in the media shown in Table 1 was detected based on the following observations: The culture filtrates showed greater activity against the β-lactam hypersensitive mutants of *Pseudomonas aeruginosa*[8] and *Escherichia coli*[9] than their corresponding parents. Each exhibited β-lactamase inhibitory activity in a convenient assay system (Table 2). Each culture filtrate induced β-lactam-specific morphological changes of the mutants (Fig. 1). However, it was surprising that the active substances were highly stable to β-lactamases (Table 2).

Fig. 2 shows that while strains YK-278 and YK-280 produced only cephabacins residing at the TLC origin in this solvent system, strain YK-90 produced not only cephabacins but also some non β-lactam antibiotics which have not been characterized.

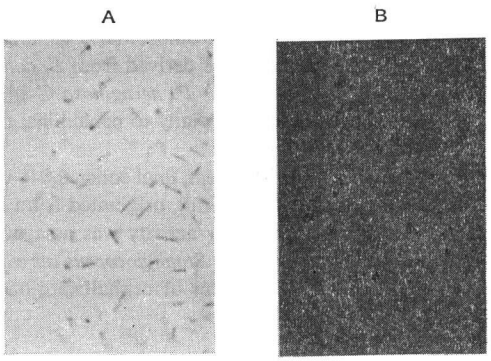
Table 1. Media used for production of cephabacins.

Seed medium (%)		Fermentation medium (%)		
			Flask	Fermentor
Glucose	2	Dextrin	3	3
Soluble starch	3	Soybean flour	1.5	3
Soybean flour	1	Corn-gluten meal	1.5	—
Corn-steep liquor	0.3	Polypepton	0.2	—
Polypepton	0.5	$Na_2S_2O_8 \cdot 5H_2O$	0.1	—
NaCl	0.3	$CaCO_3$	0.5	0.5
$CaCO_3$	0.5			
pH[a]	7.0	pH[a]	6.5	Not adjusted

[a] pH was adjusted before the addition of $CaCO_3$.

In addition, deacetylcephalosporin C was detected in methanol-extracts from cells of strains YK-278 and YK-280, but not in strain YK-90[1].

Fig. 1. Morphological change of *P. aeruginosa* C141 induced by a culture filtrate of strain YK-90. A; control, B; treated by the culture filtrate.



Taxonomy of the Producing Organisms

Strain YK-90 was isolated from a soil sample collected at Niimi City, Okayama Prefecture, Japan. Strains YK-278 and YK-280 were isolated from plant specimens obtained at Ayama District, Mie Prefecture, Japan. Unless otherwise stated, the cultivation temperature was 24°C.

Morphology

The morphological characteristics of the three strains observed after 5 days of cultivation are shown in Table 3. Strain YK-90 was Gram-negative, slender (sometimes filamentous) rods and motile by gliding. Strains YK-278 and YK-280 had similar characteristics; they were Gram-negative motile rods with a polar flagellum. These three strains did not form spores or microcysts.

Growth on Several Media

Colonies of strain YK-90 were mucoidal, nontransparent pale yellow, circular, convex with an entire edge on nutrient agar. Clear zones were formed around colonies on yeast cell agar[12]. Gelatin was liquefied, reducing activity of litmus was weak and milk was peptonized.

Colonies of strains YK-278 and YK-280 were translucent, lemon-yellow, circular, capitate with an entire edge. These strains strongly liquefied gelatin. They did not reduce litmus and only weakly peptonized milk.

The intracellular pigments of strains YK-278 and YK-280 seemed to be carotinoids since they gave a deep-blue color with concentrated sulfuric acid[13]. In contrast, that of strain YK-90 gave a purple-red color, indicating the absence of carotinoid pigments.

Physiological Characteristics

The physiological properties of the producing strains are summarized in Table 4. All these strains had high GC contents of DNA and *meso*-type diaminopimelic acid in their cell walls. They

Table 2. Antibacterial and β-lactamase inhibitory activity of culture filtrates of the cephabacin-producers.

	Inhibition-zone (mm) of culture filtrate[a] of		
	YK-90	YK-278	YK-280
Antibacterial activity[b]			
Escherichia coli CPC 20	—[c]	10.5	14
E. coli PG-12	17	10.5	14
E. coli PG-8	23.5	27	25
E. coli PG-8+penicillinase[d]	23.5	27	25
E. coli PG-8+cephalosporinase[d]	23	23	21.5
Pseudomonas aeruginosa IFO 3080	—	—	—
P. aeruginosa C 141	28	34	31
β-Lactamase inhibitory activity[e]			
St	17.5	—	—
Si	25.5	21	25.5

[a] One milliliter of seed culture grown in a test tube containing 5 ml of the seed medium (Table 1) at 24°C overnight with reciprocal shaking was transferred into a 200-ml Erlenmeyer flask containing 40 ml of the fermentation medium (Table 1). The fermentation was carried out at 17°C for 72 hours on a rotary shaker.

[b] *E. coli* CPC 20 is a chromosomal β-lactamase-defective mutant (*amp C*$^-$) derived from *E. coli* LD-2. *E. coli* PG-12 and PG-8 derived from *E. coli* CPC 20 are a permeability mutant and a PBP 1B-defective mutant, respectively[9]. *P. aeruginosa* C 141 derived from *P. aeruginosa* Css[8] is more sensitive to β-lactam antibiotics, especially to penicillins, than the parental strain.

[c] No inhibition zone.

[d] Penicillinase (Calbiochem, final concn 0.048 unit/ml), originated from *Bacillus cereus*. Cephalosporinase (final concn 0.025 unit/ml), originated from *Enterobacter cloacae*.

[e] β-Lactamase inhibitory activity was assayed by the comparison with diameters of inhibition zones on plates (St) seeded with *Staphylococcus aureus* FDA 209P and plates (Si) seeded with the same organism, containing 0.002 unit/ml of cephalosporinase derived from *Proteus vulgaris* GN4413 and 2 μg/ml of cephaloridine.

Fig. 2. Bioautograms of culture filtrates of the cephabacin-producers.

Culture filtrates (5 μl) were applied on cellulose films (Tokyo Kasei) and developed using a solvent system of CH_3CN - H_2O (4: 1).

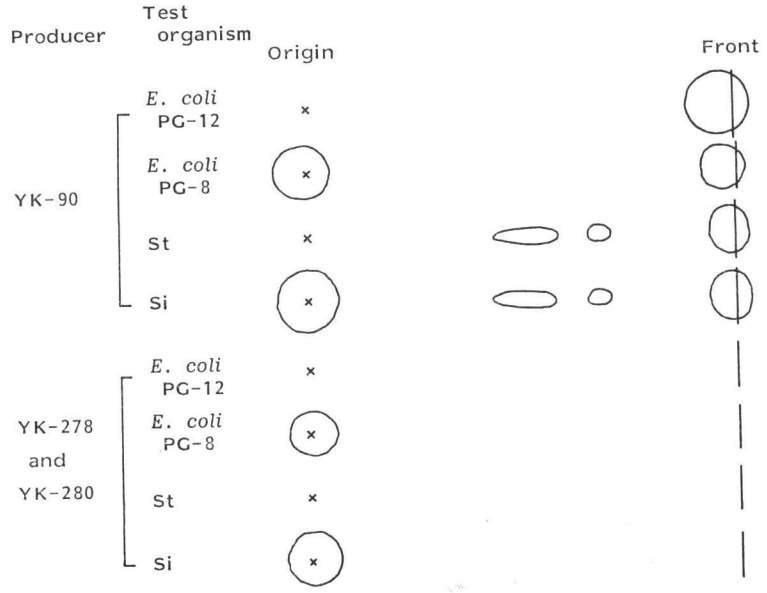


Table 3. Morphological characteristics of the cephabacin-producers.

	Strain		
	YK-90	YK-278	YK-280
Gram's stain	Negative	Negative	Negative
Size; width (μm)	0.7~0.9	0.2~0.8	0.2~0.8
length (μm)	1.0~3.5[a]	0.6~1.9	0.6~1.9
Flagellation	None	Polar	Polar
Motility[b]	Gliding	+	+
Spore formation	—	—	—
Microcyst formation[c]	—	—	—

[a] Filamentous cells of 6.5~8.5 μm length were often observed.
[b] Motility was observed according to GILARDI[10].
[c] Formation of microcyst was tested according to DWORKIN and GIBSON[11].

Table 4. Physiological characteristics of the cephabacin-producers.

	Strain		
	YK-90	YK-278	YK-280
Reduction of nitrate	—	—	—
Denitrification	—	—	—
Methyl red test	—	—	—
Voges-Proskauer test	—	—	—
Production of indole	—	—	—
Production of H_2S	—	±	+
Hydrolysis of starch	—	+	+
Utilization of citrate	+	+	+
Utilization of inorganic nitrogen sources;			
Potassium nitrate	—	+	—
Ammonium sulfate	+	±	+
Production of diffusible pigment	—	—	—
Urease	—	—	—
Oxidase	+	+	+
Catalase	+	+	+
Range of growth			
pH	5.4~7.6 (Optimum 5.6~6.6)	4.6~8.3 (Optimum 6.9~7.7)	4.6~8.3 (Optimum 6.2~7.3)
Temperature (°C)	10~30 (Optimum 15~27)	14~38 (Optimum 18~25)	11~40 (Optimum 16~26)
Oxygen demand	Aerobic~facultatively anaerobic	Aerobic	Aerobic
OF-test	Not reactive	Oxidative (Slowly)	Oxidative (Slowly)
Tolerance to NaCl (%)	0~4	0~4	0~4
Degradation of			
Colloidal chitin	+	—	+
Carboxymethylcellulose	+	+	+
Alginate	±	+	—
Agar	—	—	—
Tween 80	+	+ (Fast)	+ (Fast)
GC content of DNA (%)[a]	75.8±1.5	74.4±1.5	75.7±1.5

[a] Determined by the thermal denaturation method in ×0.1 standard saline citrate solution.

Table 5. Acid and gas formation from sugars and utilization of sugars by the cephabacin-producers.

Sugar	Strain								
	YK-90			YK-278			YK-280		
	Acid[a]	Gas[a]	Growth[b]	Acid	Gas	Growth	Acid	Gas	Growth
L-Arabinose	−	−	−	−	−	+	±	−	+
D-Xylose	−	−	−	−	−	+	−	−	+
D-Glucose	−	−	+	−	−	+	−	−	+
D-Mannose	−	−	+	−	−	±	−	−	+
D-Fructose	−	−	−	−	−	+	−	−	+
D-Galactose	−	−	−	−	−	+	−	−	+
Maltose	−	−	+	−	−	+	−	−	+
Sucrose	−	−	−	−	−	+	−	−	+
Lactose	−	−	+	−	−	+	−	−	+
Trehalose	−	−	+	−	−	+	−	−	+
D-Sorbitol	−	−	±	−	−	±	−	−	−
D-Mannitol	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−
Glycerol	−	−	−	−	−	+	−	−	+
Starch	−	−	±	−	−	+	−	−	+

[a] Acid and gas formation from sugars were examined in peptone-water containing 0.1% of a single carbon source.

[b] Sugar utilization was tested using Davis agar containing 1% of a single carbon source, supplemented with 0.005% each of L-cysteine and L-tryptophan.

showed degradative activities against some polysaccharides; strains YK-90 and YK-280 degraded colloidal chitin and carboxymethyl cellulose and strain YK-278 degraded carboxymethyl cellulose and alginate.

Acid and Gas Formation from Sugars and Utilization of Sugars

No strains formed acid or gas from any of the sugars tested (Table 5). Strains YK-278 and YK-280 assimilated a wide variety of sugars and their assimilation patterns were similar. In contrast, strain YK-90 assimilated only a few sugars.

Identification

The characteristics of these three cephabacin-producing strains were compared with those of the species described in BERGEY's Manual of Determinative Bacteriology (8th ed.) and cited in the validation lists of the International Journal of Systematic Bacteriology. The above key characteristics indicated that strain YK-90 belongs to the genus *Lysobacter* and strains YK-278 and YK-280 to the genus *Xanthomonas*. Although four species and one subspecies of *Lysobacter* have appeared in publication[14], strain YK-90 did not completely coincide with any of them and therefore was designated as *Lysobacter lactamgenus* sp. nov. YK-90.

None of the five known species of *Xanthomonas*[15] has all the following characteristics of strains YK-278 and YK-280; 1) gelatin liquefaction positive, 2) oxidase positive, 3) no acid or gas formation from sugars, and 4) positive growth in the presence of 4% sodium chloride. Strains YK-278 and YK-280 were similar to each other though there were some minor differences in such properties as the utilization of inorganic nitrogen sources and decomposition activity of some polysaccharides. We thus regarded them as the same species and designated them *Xanthomonas lactamgena* sp. nov. YK-278 and YK-280.

Fermentation

Seed culture for large-scale fermentation was performed by inoculating a loopful of cells into two 2-liter Sakaguchi flask containing 500 ml of the seed medium (Table 1) and incubating the flasks at 24°C for 48 hours on a reciprocal shaker (125 revolutions/minute). All of the seed culture was transferred into a 200-liter fermentor containing 120 liters of the seed medium supplemented with 0.05% of Actocol (an antifoam distributed by Takeda Chem. Ind.). The seed culture was incubated at 24°C for 48 hours with an agitation rate of 150 rpm and an air flow of 120 liters/minute. Forty liters of this culture was transferred into a 2,000-liter fermentor containing 1,200 liters of the large-scale fermentation medium (Table 1) supplemented with 0.05% of Actocol. The fermentation was carried out at 24°C for 66 hours with an agitation rate of 120 rpm and an air flow of 1,200 liters/minute.

Typical large-scale fermentation profiles for cephabacin production are shown in Fig. 3. As reported in the accompanying papers[1,2], the strains YK-90, YK-278 and YK-280 produced cephabacin $F_{1\sim3}$ and $H_{1\sim3}$, cephabacin $F_{4\sim9}$ and $H_{4\sim6}$ and cephabacin $F_{4\sim6}$ and $H_{4\sim6}$, respectively.

Discussion

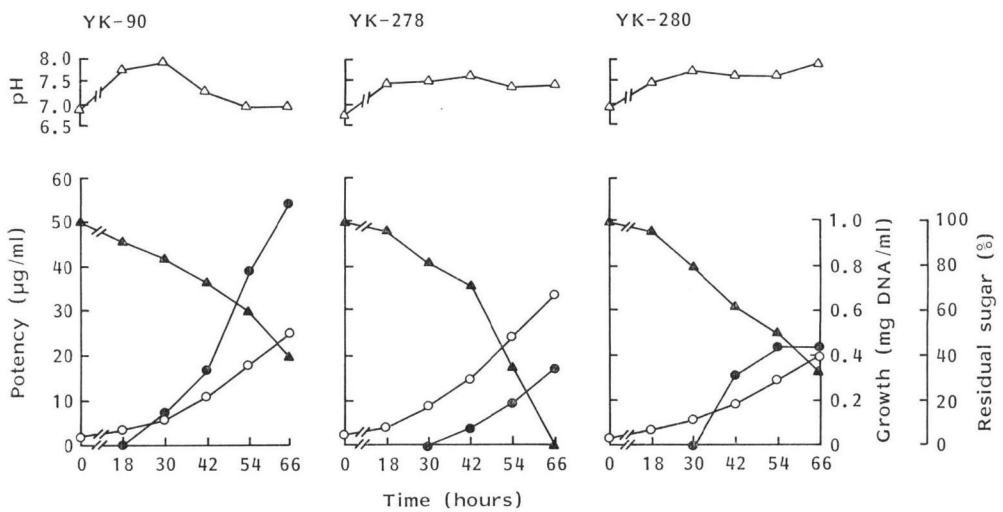
The following key characteristics of strain YK-90 have led us to identify this strain as a species of *Lysobacter*; pale yellow non-flagellated rods or filaments, gliding motility, aerobic, a high GC content of DNA, microcyst-forming ability negative, decomposition of carboxymethyl cellulose and colloidal chitin positive, lytic activity against dried yeast cells positive and mucoidal growth on skim milk - acetate agar[17]. The family *Lysobacteriaceae* which consists of one genus, *Lysobacter*, can be distinguished from the two related families of gliding bacteria, *Cytophagaceae* and *Myxobacterales*, by the GC content of DNA and the microcyst-forming ability, respectively[14]. The genus *Flexibacter* belonging to gliding bacteria is known to produce monobactam antibiotics[18,19], but the genus *Lysobacter* described

Fig. 3. Time-course of large-scale fermentation for cephabacin production.

Potency (●). Calculated as cephabacin F_1. The β-lactam hypersensitive mutants[8,9] were used as test organisms.

Growth (○). The DNA content was determined by the method of BURTON[16] after extraction with 5% perchloric acid.

Residual sugar (▲), pH (△). Determined by the glucose oxidase method.



here is the first member of gliding bacteria producing cephem antibiotics.

On the other hand, the characteristics of YK-278 and YK-280 such as motile yellow rods with a polar flagellum, carotinoid pigment positive, aerobic, a high GC content of DNA, nitrate reduction negative, fast decomposition of starch and Tween 80, suffice to designate YK-278 and YK-280 as species of *Xanthomonas*. Although these strains differed from each other in some physiological characteristics described in the text, and susceptibilities to polymyxin B and chlortetracycline among the 18 antibiotics tested (data not shown), they belong to the same species.

Squibb researchers have reported that several strains of *Flavobacterium* and *Xanthomonas* produced deacetoxycephalosporin C in their cells[6] and have recently shown that one of the *Flavobacterium* strains also produced two 7-formamidocephalosporins in its culture filtrate[7]. We have obtained 24 strains producing the related cephem antibiotics, cephabacins, which included 11 strains of the YK-90 type, 3 strains of the YK-278 type and 10 strains of the YK-280 type. The three typical strains produced cephabacin $F_{1\sim3}$ and $H_{1\sim3}$, cephabacin $F_{4\sim9}$ and $H_{4\sim6}$ and cephabacin $F_{4\sim6}$ and $H_{4\sim6}$, respectively[1,2]. *X. lactamgena* YK-278 and YK-280 seem to differ from *Xanthomonas* SC 11,696 which was not fully described[6], the producer of deacetoxycephalosporin C, because the latter is oxidase negative, forms acid from glucose, arabinose and cellobiose, and has no carotinoid pigment.

Since 1981, various types of β-lactam antibiotics including monobactams[18~23], a carbapenem antibiotic[24] and cephalosporins[6,7] have been detected from many taxonomically different bacteria. These findings have led to the conclusion that bacteria possess the ability to synthesize the β-lactam nucleus.

Although the biosynthesis of the cephabacin antibiotics is not clear, it is interesting in their biosynthetic pathway, especially in regard to the 7-formylamino substituent and the 3-side chains.

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