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Effects of Amino Acids on the Amidation of Polyaromatic Carboxylic Acids by *Bacillus cereus*

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The soil bacterium *Bacillus cereus* Tim-r01 efficiently transformed polyaromatic carboxylic acids (PACA) such as 4-biphenylcarboxylic acid (4-BPCA), 4-biphenylacetic acid, and 4-phenoxybenzoic acid into their corresponding amides. The amidation activity was expressed at 37°C (pH 7–8) in the presence of grown cells in nutrients under an aerobic atmosphere. Other strains of *B. cereus*, IFO 3001 and IAM 1229, also gave the amide from 4-BPCA. In phosphate-buffered saline (PBS), the addition of normal amino acids was essential, while sulfur-containing amino acids such as methionine and cysteine drastically inhibited the amidation. Tracer experiments using N-15-isoleucine and N-15-alanine showed that the nitrogen atom of the amide came from an amino group of amino acids but not from ammonia or alkylamines.

Key words: *Bacillus cereus*; biotransformation; amidation; 4-biphenylcarboxylic acid; polyaromatic carboxylic acid

Polyaromatic carboxamides, widely used in fine chemical industries for the syntheses of pesticides, pharmaceuticals, and liquid crystals, are chemically prepared by heating PACA with ammonia under anhydrous conditions or by treating acid chlorides with ammonia. On the other hand, the transformation of nitrile to amide in the presence of nitrile hydratase has been done on an industrial scale under mild conditions.[1)]

Recently, natural carboxylic acids have been transformed into amides by the use of microorganisms. Examples are the amidation of biotin to biotinamide by *Rhodotorula flava*,[2)] 12-hydroxyoctadecanoic acid to 12-hydroxyoctadecanamide by *Bacillus cereus* 50,[3)] and oleic acid to octadecenamide by *Bacillus megaterium* NRRL B-3437 and *Bacillus cereus* NRRL-14812.[4,5)]

During the screening of bacteria, we found that the transformation of 4-BPCA to 4-phenylbenzamide (4-PBAm) took place in the presence of *B. cereus*, a wild-type Tim-r01, in good yield without the use of organic solvents.[6)] Since bacteria grow fast and are easily dispersed in an aqueous medium by shaking, whole cells can be conveniently used as biocatalysts. In this study, the properties of *B. cereus* and the effects of amino acids on the amidation of PACA were investigated. The N-source of 4-PBAm was also investigated by using N-15 isoleucine and N-15 alanine as tracers of nitrogen atoms.

Materials and Methods

Chemicals. 4-BPCA, *N*-phenylanthranilic acid, 4-carboxy-4′-hydroxybiphenyl, and 2-naphthoic acid were purchased from Wako Pure Chemicals Co. 4-Biphenylacetic acid and 9-fluorenone-2-carboxylic acid were obtained from Tokyo Kasei Co. 4-Phenoxybenzoic acid was kindly obtained from Nihon Nohyaku Co. N-15-Isoleucine and N-15-alanine were purchased from Aldrich Chem. Co. Solvents and other chemicals and reagents were of the highest purity available.

Microorganisms. A bacterium (Tim-r01) strongly active in the amidation of 4-BPCA was isolated from soil in Toyama Prefecture, Japan. The species was identified as *B. cereus* according to Burgey's manual[7)] and finally by the BIOLOG system. *B. cereus* is a facultatively anaerobic, spore-forming, mesophilic, and rod-shaped bacterium. *B. cerues* IFO 3001, IFO 15305, IFO 13466, IFO 3563, IFO 3836, and IFO 13494, *B. subtilis* IFO 3335, and *B. brevis* IFO 15304 were obtained from the Institute for Fermentation, Osaka (IFO). *B. megaterium* ACCC 10011 was from the Agricultural Culture Collection, China (ACCC).

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IAM 1110, IAM 1029, IAM 1229, IAM 1656, and IAM 1729 were kindly provided from the Institute of Applied Microbiology, (IAM). Baker's yeast was purchased from a grocery.

Medium and cultivation. The basal medium used in our experiments contained (per liter) polypeptone (10 g), bonito extract (10 g), and sodium chloride (2 g). Before autoclaving, the pH of the medium was adjusted with HCl and NaOH solution to 7.4. Bacterial strains were inoculated into 10 ml of medium in an L-shaped test tube (30 ml) and shaken at 30–37°C for 12 h.

In larger-scale experiments, the cultured solution was added to a 500-ml Sakaguchi flask containing 200 ml of the medium and shaken reciprocally at 120 rpm for 24 h. Cells were harvested by centrifugation ($3000 \times g$, 15 min) and used as a biocatalyst.

Amidation and analysis of products. An L-shaped test tube (30 ml) containing 0.2 g of fresh cells, 1–2 mM of substrate dissolved in 0.1 ml of DMSO, and 10 ml of the medium was shaken at 37°C for 24 h. After the reaction, the solution was acidified with 1 ml of 2 M HCl and extracted three times with 2 ml of ethylacetate. The upper layer was carefully pipetted out, collected, and analyzed by high-pressure liquid chromatography (HPLC) (Gilson Co. model 102, column YMC-Pack, ODS-A; 4 mm in i.d. × 150 mm in length) with a gradient system (methanol:water = 3:7 to 7:3) at 270 nm.

Isolation of products. To a 500-ml Sakaguchi flask was added 200 ml of medium, 2 wet g of bacteria, and 2 mM of substrate. The flask was shaken at 120 rpm at 37°C for 2 days. After the reaction, the bacterial solution was alkalified with NaOH and extracted with ethylacetate. The extracts were washed with a saturated aqueous solution of NaCl and dried over anhydrous Na_2SO_4. After filtration, the solvent was concentrated in a rotary evaporator under decreased pressure. The residue was then purified by a preparative HPLC (Jasco Co., column, YMC-pack ODS; 150 mm in length × 20 mm in i.d., methanol:water = 80:20) and analyzed by ^{1}H-NMR, ^{13}C-NMR, MS spectra, IR spectra, and elementary analysis. The melting point of the product was compared with those in the literature.

Results and discussion

Amidation of PACA

The amidation of 4-BPCA in the presence of several species and strains of bacteria was examined. Results are shown in Table 1. Among 12 strains of *B. cereus*, two strains (Tim-r01 and IFO 3001) were highly active in the amidation of 4-BPCA. *B. megaterium* ACCC 10011, *B. subtilis* IFO 3335, *B. brevis* Tim-r02 (wild type),[8] *E. coli* XL1 Blue, and baker's yeast were inactive.

Table 1. Amidation of 4-BPCA in the Presence of Various Microorganisms

Bacterium	strain	yield (%)
None		0
Bacillus cereus	Tim-r01	75 ± 5
	IFO 3001	76 ± 9
	IAM 1029	71 ± 3
	IAM 1110	11 ± 6
	IAM 1656	7 ± 1
	IAM 1229	4 ± 3
	IAM 1729	2 ± 2
	IFO 15305	0
	IFO 13466	0
	IFO 3563	0
	IFO 3836	0
	IFO 13494	0
Bacillus brevis	Tim-r02	0
Bacillus megaterium	ACCC 10011	0
Bacillus subtilis	IFO 3335	0
Echerichia coli	XL1 Blue	0
Baker's yeast	..	0

Amidation was done in the presence of bacterial cells (0.2 wet-g) or baker's yeast (0.2 g) by the addition of 4-BPCA (2 mM) in a fresh culture at 37°C for 24 h. The table represents the averages and standard deviations of three independent measurements.

Several substrates were then made into their corresponding amides by *B. cereus* Tim-r01, in moderate to excellent yields, in basal medium at 37°C for 24 h: 4-BPCA (95%), 4-biphenylacetic acid (67%), 4-phenoxybenzoic acid (69%), 4-carboxy-4′-hydroxybiphenyl (52%), *N*-phenylanthranilic acid (57%), 9-fluorenone-2-carboxylic acid (41%), and 2-naphthoic acid (17%).[6] Optimum conditions for growth of *B. cereus* Tim-r01 were pH 7-8 and a temperature between 30–37°C. Doubling time of the growth of bacteria in an exponential phase was 20 min at 35°C. The density of bacteria in the medium was saturated after 8 h. The optimal conditions in our amidation coincided with the optimal growth conditions of *B. cereus* Tim-r01, suggesting that the amidation competes with the metabolism of these microorganisms. The yield vs. reaction time in the amidation of 4-BPCA by *B. cereus* Tim-r01 is shown in Fig. 1. Amidation began after the growth of bacteria had reached a stationary phase (8 h), and fast amidation continued until a 77% yield (24 h) was attained. The pH of the reaction solution gradually increased by evolution of ammonia as a result of the decomposition of polypeptone. When grown cells (0.2 g) in nutrient broth (10 ml) and 2 mM of substrate were used, no induction period was observed and the yield reached 90% at 37°C after 18 h. An aerobic atmosphere was considered to be indispensable for the amidation since amidation under an N_2 or CO_2 atmosphere gave a poor yield (Table 2).

Physical and spectral properties of amides ob-

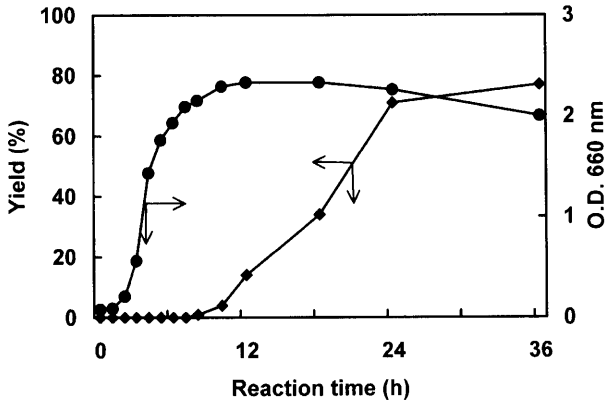

Fig. 1. Growth of Bacteria and Amidation of 4-BPCA.
Cultivation of bacteria and amidation of 4-BPCA were done in an L-shaped test tube (10 ml medium, 0.1 ml cultured solution, and 1 mM 4-BPCA as a substrate) at 37°C. The growth of bacteria was monitored by optical density at 660 nm. The figure represents the averages of three independent measurements.
●: Yield (%), ▲: O.D. 660 nm

Table 2. Amidation of 4-BPCA by *Bacillus cereus* Tim-r01

4-BPCA (mM)	atmosphere	yield (%)
1.0	air	95±3
1.0	N_2	1±1
1.0	CO_2	0
2.0	air	75±5
3.0	air	34±9

Amidation was done in 10 ml of a basal medium of bacterial cells in the presence of 0.2 wet-g and 4-BPCA dissolved in DMSO (0.1 ml) at 37°C for 24 h. The table represents the averages and standard deviations of at least three independent measurements.

Table 3. Amidation of 4-BPCA in the Presence of Various Nitrogen Sources

Nitrogen source	concentration (mM)	yield (%)
Control	—	19±10
$(NH_4)_2SO_4$	10	16±13
NH_4Cl	20	21±17
NH_4HCO_3	20	22±13
Glycine	20	68±10
Methylamine	10	0
Ethylamine	10	0
Peptone	2%	79±10

After nitrogen sources had been added, pH of PBS was readjusted to 7.0. Grown cells were collected and washed three times with PBS. To PBS (10 ml) containing 2 mM 4-BPCA, *B. cereus* Tim-r01 (0.2 wet-g) and a nitrogen source were added. The reaction was done at 37°C for 24 h. The table represents the averages and standard deviations of three independent measurements.

tained from PACA are as follows:

4-PBAm; mp. 233-234°C, Lit. 221-223°C.[9] ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.97-7.36 (9H, m, aromatic). MS m/z: 197 (75) $(M)^+$, 181 (100) $(M-NH_2)^+$, 153 (47.5) $(M-CONH_2)^+$. IR 3408 and 3190 cm^{-1}: antisymmetric or symmetric stretch of amide (NH_2), 1645 cm^{-1}: C=O stretch of primary amide. Anal. found: C, 79.27; H, 5.77; N, 6.84%. Calcd. for $C_{13}H_{11}NO$: C, 79.16; H, 5.62; N, 7.10%.

4-Biphenylacetamide; mp. 241-243°C, Lit. 242.5-243.5°C.[10] ^{1}H-NMR (400 MHz DMSO) δ: 7.65-7.34 (9H, m, aromatic), δ: 3.41 (1H, s, CH_2). MS m/z: 211 (46) (M^+), 167 (100) $(M-CONH_2)$. IR 3350 $cm^{-1}$and 3180 cm^{-1} (amide NH_2), 1640 cm^{-1} (amide C=O). Anal. found: C, 79.91; H, 5.87; N, 6.52%. Calcd. for $C_{14}H_{13}NO$; C,79.59; H,6.20; N,6.63%.

N-Phenylanthranilamide; mp. 127-128.5°C, Lit. 127.5-129°C.[11] ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.66-6.76 (9H, m, aromatic). MS m/z: 212 (49) $(M)^+$, 195 (100) $(M-NH_2-H)^+$, 167 (43) $(M-CONH_2-H)^+$. IR 3350 and 3178 cm^{-1}: antisymmmetric or symmetric stretch of amide (NH_2), 1652 cm^{-1}: C=O stretch of primary amide. Anal. Found: C, 73.56; H, 5.81; N, 12.85%. Calcd. for $C_{13}H_{11}N_2O$; C, 73.56; H, 5.70; N, 13.20%.

4′-Hydroxybiphenyl-4-carboxamide; mp. 273-275°C. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.91-6.86 (8H, m, aromatic). MS m/z: 213 (100) $(M)^+$, 197 (100) $(M-NH_2)^+$, 169 (20) $(M-CONH_2)^+$. IR 3405 and 3190 cm^{-1} (NH_2 amide), 1645 cm^{-1} (C=O amide). Anal. found: C,73.11; H,5.30; N,6.01%. Calcd. for $C_{13}H_{11}NO_2$: C, 73.22: H, 5.20: N, 6.57%.

9-Fluorenone-2-carboxyamide; mp. 255-256°C, Lit. 252-254°C.[12] ^{1}H-NMR (400 MHz, DMSO) δ: 8.15-7.42 (9H, m, aromatic). MS m/z: 223(80)$(M)^+$, 207 (100) $(M-NH_2)^+$, 179 (26) $(M-CONH_2)^+$, 151 (51) $(M-CONH_2-CO)^+$. IR 3370 and 3180 cm^{-1} (amide NH_2), 1650 cm^{-1} (C=O amide). Anal. found: C, 74.84; H, 4.15; N, 5.75%. Calcd. for $C_{14}H_9NO_2$: C, 75.32; H, 4.06; N, 6.28%.

Nitrogen source of amide

The nitrogen source for the amidation of 4-BPCA was investigated in PBS in the presence of various nitrogen compounds. After the growth curve of *B. cereus* Tim-r01 had reached a plateau, the cells were harvested with a centrifuge and washed three times with PBS, and then compounds such as alkylamines, ammonium salts, glycine, and peptone were respectively added to a bacterial PBS solution (Table 3).

The 19% yield of 4-PBAm in the control experiment suggested that the nitrogen atom of 4-BPAm came from the cell components of the bacteria themselves. The addition of alkylamines strongly disturbed the amidation. Interestingly, the addition of glycine and peptone increased the yield, while the addition of ammonia gave almost the same yield as that of the control experiment. Reproduction of the amidation yield showed significant differences. Therefore, the mean of the values and standard deviation were measured from three or more independent experiments.

The effects of 20 kinds of amino acids on the yields of amide were compared (Table 4). The yield in-

Table 4. Effects of Amino Acids on Amidation of 4-BPCA

Amino acid	yield (%)
Control	19±4
L-Histidine	71±12
L-Isoleucine	63±1
L-Glutamine	63±9
L-Asparagine	57±9
Glycine	53±9
L-Glutamic acid	44±7
L-Leucine	42±7
L-Alanine	40±16
L-Aspartic acid	38±6
L-Threonine	37±17
L-Proline	37±9
L-Arginine	33±7
L-Lysine	30±5
L-Serine	30±17
L-Valine	29±3
L-Phenylalanine	21±1
L-Tyrosine	20±5
L-Tryptophan	20±3
L-Methionine	4±3
L-Cysteine	1±1

Grown cells were collected and washed three times with PBS. To PBS (10 ml), 2 mM 4-BPCA, *B. cereus* Tim-r01 (0.2 wet-g), and amino acid (10 mM) as a nitrogen source were added, and the pH of PBS was readjusted to 7.0. The reaction was done at 37°C for 24 h. The table represents the averages and standard deviations of at least three independent measurements.

creased or decreased according to the type of amino acid. The addition of L-histidine gave the highest yield. Amino acids having an aromatic group, such as L-phenylalanine, L-tryptophan and L-tyrosine, had no substantial effect. On the other hand, glycine, L-alanine, L-valine, L-isoleucine, L-serine, L-threonine, and L-asparagine gave moderate yields. Nutrients such as peptone are also a candidate for the nitrogen source of amidation. Interestingly, sulfur-containing amino acids, L-methionine and L-cysteine, drastically inhibited the transformation. Although the precise mechanism is not clear, sulfur-containing amino acids might have inhibited the enzymatic system for the amidation.

To determine whether amino acids are a nitrogen source, amidation of 4-BPCA was done by adding N-15-alanine or N-15-isoleucine in the presence of *B. cereus* Tim-r01. In MS analyses of 4-PBAm after extraction and purification, molecular mass ion (m/z) 198 of N-15-4-PBAm, in contrast to (m/z) 197 from none N-15 labeled 4-PBAm, and the distinct fragment ion (m/z) 181 were observed as major peaks in both cases. Therefore, in the amidation of 4-BPCA by *B. cereus*, an amino group of amino acid in nutrients was transferred to 4-BPCA. The amidation of 4-BPCA was irreversible since the substrate was not induced from 4-PBAm under the same reaction conditions. The amidation using grown cells and a fresh medium gave good results. These results also suggest that there is competition in consumption of nutrients for the growth of bacteria and the amidation.

In the amidation of oleic acid by *B. megaterium* NRRL B-3437, ammonium chloride as a nitrogen source was necessary for amide production.[4] L-Asparagine was obtained from L-aspartic acid, ammonia, ATP, and magnesium ion in the presence of L-asparagine synthetase by way of aminoacyladenylate as an intermediate.[13,14] Carbamoylphosphate synthetase amidotransferase from *E. coli* catalyzed the formation of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of ATP.[15] These results indicate that enzymatic amidation needs both nitrogen and energy sources. In our amidation, construction of an amidation system in 4-BPCA from the homogenized cell components of *B. cereus* and ATP was not successful. Further investigation of the enzyme system and identification of related genomes of this bacterium is in progress in our laboratory.

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References

1) Mettler, H. -P., Three-step method for the preparation of 4'-hydroxy-biphenyl-4-carbonitrile from 4-hydroxybiphenyl. *Ger. Offen* 19833409 (1998), *Chem. Abstr.*, **130**, 66276 (1999).
2) Sekijo, C., Tsuboi, Y., Yoshimura, Y., and Shoji, O., Studies on production of biotin by microorganisms. *Agr. Biol. Chem.*, **32**, 1181–1186 (1968).
3) Huang, J. -K., Keudell, K. C., Klopfenstein, W. E., Wen, I., Bagby, M. O., Vesonder, R. F., Norton, R. A., and Weisleder, D., Biotransformation of 12-hydroxyoctadecanoic acid to 12-hydroxyoctadecanamide by *Bacillus cereus* 50. *J. Am. Oil Chem. Soc.*, **74**, 601–603 (1997).
4) Kaneshiro, T., Vesonder, R. F., Peterson, R. E., Weisleder, D., and Bagby, M. O., *J. Am. Oil Chem. Soc.*, **71**, 491–494 (1994).
5) Kaneshiro, T., Nakamura, L. K., and Bagby, M. O. Oleic acid transformations by selected strains of *Sphingobacterium thalpophilum* and *Bacillus cereus* from composted manure. *Curr. Microbiol.*, **31**, 62–67 (1995).
6) Maruyama, R., Ono, S., and Inoue, M., Amidation of polyaromatic carboxylic acids in aqueous medium. *Tetrahed. Lett.*, **41**, 5229–5232 (2000).
7) Sneath, P. H. A. in Burgey's manual ed, by Holt, J. G., *Williams & Wilkins*, pp. 1104–1141, (1986).
8) Inoue, T., Tasaka, T., Maruyama, R., Sumita, Y., Ono, S., and Inoue, M., Bacterial reduction of 9-fluorenone to 9-hydroxyfluorene. *Biosci. Biotechnol. Biochem.*, **62**, 814–815, (1998).
9) Gbrook, A. G., Cohen, H. L., and Wright, G. F., Reaction of the stilbene-disodium and -dilithium adducts. *J. Org. Chem.*, **18**, 447–463 (1953).

10) DeTar, D. F., and Carmack, M., A study of reaction conditions with acetophenone and other ketones. *J. Am. Chem. Soc.*, **68**, 2025–2029 (1946).
11) Goodmann, M., Arbeiter, N., and Powell, G., The anhydrides of N-arylanthranilic acids. *J. Am. Chem. Soc.*, **55**, 4294–4297 (1933).
12) Hodson, H. F., Batchelor, J. F., and Gorvin, J. H., Cyclic carbonyl compounds. *Ger. Offen.* 2243997 (1973), *Chem. Abstr.*, **85**, 123668 (1973).
13) Cedar, H., and Schwartz, J. H., The asparagine synthetase of *Echerichia coli*, I. Studies on mechanism. *J. Biol. Chem.*, **244**, 4122–4127 (1969).
14) Cedar, H., and Schwartz, J. H., The asparagine synthetase of *Echerichia coli*, II. Biosynthetic role of the enzyme, purification, and characterization of the reaction products. *J. Biol. Chem.*, **244**, 4112–4121 (1969).
15) Huang, X., and Raushel, F. M., Deconstruction of the catalytic array within the amidotransferase subunit of carbamoyl phosphate synthetase. *Biochemistry*, **38**, 15909–15914 (1999).