A novel esterase showing activity specific for esters of aryl-carboxylic acids was discovered in Sporosarcina sp. nov., which was identified by the 16S rDNA sequencing method in addition to morphological and physiological analyses. The aryl-carboxylesterase (named EstAC) was purified 780-fold from crude cell extracts by a 5-step procedure. EstAC was characterized as a monomeric protein with a molecular weight of 43,000, an optimum pH of around 9.0, and an optimum temperature of 40°C. The pH optimum and the effects of inhibitors together with an internal amino acid sequence suggested that EstAC is a member of family VIII esterases. EstAC was found to be highly active on a wide variety of substrates such as alkyl benzoates, alkyl phenylacetates, ethyl α- or β-substituted phenylpropanoates, dialkyl terephthalates, dimethyl isophthalate, and ethylene glycol dibenzoate. However, monomethyl terephthalate was not hydrolyzed. It was suggested that EstAC had 4-hydroxybenzoyl and cinnamoyl esterase activities as well.

**Key words:** aryl-carboxylesterase; benzyl esterase activity; diethyl terephthalate; substrate specificity; *Sporosarcina* sp.

Esterases (EC 3.1.1.x) are ubiquitous hydrolases in all of the kingdoms of life. They catalyze the hydrolysis and formation of ester bonds and act on a wide variety of natural and xenobiotic substrates. Carboxylesterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are two major classes of esterases. Both enzymes are relatively stable in organic solvents and have numerous applications in organic synthesis. They are useful catalysts for regio- and stereoselective reactions in fine-chemical reactions, and also used as bulk enzymes in a variety of chemical and other industries. In general, the esterases are specific for either the alcohol or the acid moiety on the substrate, but not for both. Many carboxylesterases have specificity for the relatively short-chain acid moiety of the carboxylic ester, different from almost all the lipases that are specific for the long-chain acid moiety or the alcohol one. The substrate specificities of a large number of carboxylesterases have been documented.

Reports on aryl-carboxylesterases showing activities for the esters of aryl-carboxylic acids containing an aromatic ring next to the ester carboxyl group, such as ethyl benzoate (EBz), are very limited compared with those on ordinary aliphatic carboxylesterases. The enzymes hydrolyzing phthalic acid esters, which are potential environmental pollutants, may be one exceptional aryl-carboxylesterase studied in many research groups. In addition, the aryl-carboxylesterases thus far reported appeared to have rather narrow substrate specificity, or the substrates studied with the enzymes are limited in structure, when one considers the occurrence of a large number of structurally analogous aryl-carboxylic acid esters. Enzymes hydrolyzing 4-hydroxybenzoic acid esters are considered to belong to a different esterase family from benzoyl esterases. Aryl-carboxylesterases, however, have many potential applications in various fields. They are attractive biocatalysts for the production of various aromas, fragrances, preservatives, and in the field of health and other industries.

Here we report a characterization of strain eSP04, a soil bacterial strain that produces an esterase capable of hydrolyzing a wide variety of aryl-carboxylic acid esters with substantial activity. We also characterize this aryl-carboxylesterase, focusing on its substrate specificity.

**Materials and Methods**

**Chemicals.** Three substrates, ethyl 2-(4-isobutylphenyl)propionate, ethyl 2-methyl-3-phenylpropionate, and ethyl 3-phenylbutanoate, were prepared from the corresponding carboxylic acids and ethanol using H$_2$SO$_4$ as a catalyst. The products were washed, evaporated, and purified by silica gel column chromatography (hexane:ethyl acetate = 20:1). 1-Phenoxy-2-propyl acetate was synthesized by esterification of 1-phenoxy-2-propanol with acetic anhydride and was distilled under reduced pressure (92°C/1.0 mmHg). The structures of the four substrates were ascertained using proton nuclear magnetic resonance (1H-NMR) spectra in CDC$_3$, with tetramethylsilane recorded on a FT NMR spectrometer (JNM-LA400, JEOL, Tokyo) at 400 MHz. All other chemicals used, including the other substrates examined, were from commercial sources.

**Media and culture conditions.** A dozen bacterial strains, maintained in our laboratory’s collection for general screening to find organisms...
having esterase activity, were examined for the hydrolysis of dihydroxy terephthalate (DET). They were cultured on DET minimal medium modified from Kurane et al. as follows: 2 g of DET, 1 g of (NH₄)₂SO₄, 1.6 g of KH₂PO₄, 0.2 g of KH₂PO₄, 0.2 g of MgSO₄·7H₂O, 0.1 g of NaCl, 20 mg of CaCl₂·2H₂O, 0.1 g of P呲 lure A2100 (a detergent, Da-ichi Kogyo Seiyaku, Kyoto, Japan), and 15 g of agar per liter (pH 7.5), supplemented with 20 mL of metal I and 10 mL of vitamin solutions. The metal I solution contained 0.5 g of FeSO₄·7H₂O, 25 mg of CuSO₄·5H₂O, 25 mg of MnSO₄·5H₂O, 25 g of ZnSO₄·7H₂O, 25 mg of Na₂MoO₄·2H₂O, and 25 mg of Na₂WO₄·2H₂O per liter; and the vitamin solution consisted of 0.2 g of myo-inositol, 0.2 g of biotin, 50 mg of vitamin B₁₂, 40 mg of calcium pantothenate, 40 mg of nicotinic acid, 40 mg of pyridoxine hydrochloride, 40 mg of thiamine hydrochloride, 40 mg of riboflavin, and 20 mg of p-aminobenzoic acid per liter (vitamins from Nacalai Tesque, Kyoto, Japan). The strain was found to form a transient halo around its colony through incubation for 2 d at 37 °C. This strain, eSP04, was selected as source of a new carboxylesterase after confirmation of its hydrolyzing ability toward EBz as well. Subculture of the strain was conducted for 5 d at 35 °C with 100 mL of fresh DET minimal medium, replacing the vitamin solution and the detergent with 0.3 g·L⁻¹ yeast extract (Nacalai Tesque) in a 500-mL Sakaguchi flask containing several stainless steel coils, as described previously, and this was repeated sequentially for 5 months.

For enzyme preparation, cells of the strain inoculated on Luria-Bertani (LB) agar plates (0.5% NaCl instead of 1%: 10 g of peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter) were grown for 16 h at 37 °C. The broth was inoculated into 2 L of the same fresh medium in a 5-L jar fermentor for 6 h at 37 °C, 300 rpm, and 2 L min⁻¹ aeration. After the addition of 5 g of DET to the culture, incubation was continued for a further 13 h. The soluble starch media was composed of 10 g of soluble starch, 5 g of peptone, 5 g of yeast extract, 1 g of KH₂PO₄, 0.2 g of MgSO₄·7H₂O, and 2 mL of metal II solution per liter (pH 8.2). The metal II solution was composed of 2.5 g of CuSO₄·5H₂O, 2.5 g of MnCl₂·4H₂O, 1.0 g of FeSO₄·7H₂O, and 0.5 g of ZnSO₄·7H₂O per liter.

Characterization of the bacterium. The strain was examined physiologically, analyzed morphologically with a scanning electron microscope (XL20, Philips Electron Optics, Eindhoven, Netherlands), and 16S rRNA gene analysis was performed for its taxonomical identification. The culture was heated for 10 min at 95 °C and total genomic DNA was extracted. The 16S rDNA sequence of the bacterium was amplified by polymerase chain reaction (PCR) using universal bacterial primers 27F (5'-AGAGTTTGTATCMTGCGCTACG-3') and 1492R (5'-TACCTTGTTACGACTCACTATAG-3'). The 5' end from position 75 to 682 (E. coli numbers) of the 16S rRNA gene was sequenced on both strands and compared with the bacterial gene sequences at DDBJ/GenBank. Sequence homology searches were performed with the BLAST program. A phylogenetic tree was constructed by the neighbor-joining method using MEGA (version 5) software.

Purification of the enzyme. Purification of the enzyme from strain eSP04 cells was conducted by ammonium sulfate precipitation and then a four-step column chromatography procedure. All procedures were carried out at 4 °C throughout purification, and esterase activity was monitored with EBz as substrate. The washed cells were suspended in 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM EDTA, disrupted using a sonic oscillator (Model 201M, Kubota, Tokyo) at 9 kHz and 150 W for 10 min, and centrifuged at 12,000 × g for 30 min. The precipitate obtained through fractionation with (NH₄)₂SO₄ (33–60% saturation) was dissolved in 25 mM of the above Tris–HCl buffer, dialyzed against the same buffer, and applied to ion-exchange chromatography (BioLogic HR System, BioRad Laboratories, Munich, Germany) on a DEAE Sepharose Fast Flow column (2.6 × 20 cm, GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The active fractions eluted with a linear gradient of NaCl (150–250 mM) were collected, brought to 15% (NH₄)₂SO₄ saturation, and applied to a TSKgel Phenyl 650-S column (2.6 × 6.4 cm, Tosoh, Tokyo) that had been equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 20% saturated (NH₄)₂SO₄. The column was washed with the same buffer containing 10% saturated (NH₄)₂SO₄, and then the active fractions were eluted with a linear gradient of (NH₄)₂SO₄ (10–2% saturation), dialyzed against 25 mM Tris–HCl buffer (pH 8.0), and concentrated by ultrafiltration (Centricon Plus-20, Millipore, Bedford, MA). The concentrate was put on a DEAE Sepharose Fast Flow column (5.75 × 75 mm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 120 mM NaCl, and then eluted with a linear gradient of NaCl (120–200 mM). The active fractions were concentrated by ultrafiltration, and were designated partially purified enzyme.

The purified enzyme was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli and stained with Coomassie Brilliant Blue R-250. Gel filtration for molecular weight determination was performed on the BioLogic HR System using a Superdex 75 pg column in 20 mM Tris–HCl buffer containing 100 mM NaCl.

Enzyme and protein assays. Esterase activities were determined by measuring the initial rates of hydrolysis of the substrates. Initial rate measurements were carried out using a pH-stat automation system (718 STAT Titrino Autotitrator, Radiometer, Lyon, France), as described previously, with some modifications. The substrate solution consisted of a suitable amount of each substrate, dimethyl sulfoxide (DMSO) in which the substrate was first dissolved at 30 °C or a higher temperature, and then mixed into 25 mM Tris–HCl buffer (pH 8.0). After 10 min of incubation of the solution at 35 °C, the reaction was started by the addition of an enzyme solution (0.5 mL) containing 0.1% bovine serum albumin (BSA) and 10 mM EDTA at a stirring rate of about 400 rpm. In the case of insoluble substrates, agitation was conducted at more than 700 rpm to achieve a sufficiently emulsified reaction mixture. The total volume of the reaction solution, and the concentrations of the substrate and DMSO at the starting point of the reaction, were adjusted to 15.0 mL, 2 or 4 mM, and 5% respectively, as per the solubility of the substrate, irrespective of the presence or absence of inhibitor or detergent. The carboxylic acid liberated from the substrate was titrated with 20–50 mM NaOH back to the initial pH of 8.0 unless otherwise stated. Spontaneous hydrolysis was subtracted at every run, although it was not substantial.

N-terminal and internal amino acid sequencing. The purified enzyme was blotted onto a polyvinylidene difluoride membrane (BioRad Laboratories), and its N-terminal amino acid sequence was determined by the Edman degradation method using a protein sequencer (Model 492 Precise Sequencing System, Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. The purified enzyme was digested with Staphylococcus aureus V8 protease (Roche, Mannheim, Germany), and the resulting peptides were separated by SDS–PAGE. The resulting major peptide, molecular weight 33,000 on SDS–PAGE, was subjected to amino acid sequencing as described above. The sequences determined were compared with protein sequence databases using the BLAST program.

Accession numbers. Strain eSP04 has been deposited in the collection of the NITE Biological Resource Center (Chiba, Japan) as strain NBRC 108908. The 16S rDNA sequence of the isolate eSP04 has been deposited in DDBJ/GenBank under accession no. AB700599.
Results and Discussion

Identification of the bacterium

Bacterial strain eSP04 that produced a DET- and EBz-hydrolyzing enzyme (an aryl-carboxylesterase, EstAC) was isolated from an enrichment culture with DET. The culture supernatant of the strain did not hydrolyze either DET or EBz, indicating that EstAC is an intracellular enzyme. The sequential subculture with DET minimal medium appeared to contribute to stabilizing the enzyme production. DET was consumed during the 5-d culture.

Morphological and physiological analyses of strain eSP04 are summarized in the left column of Table 1, which shows atypical results for several likely candidate species. The bacterium was further characterized taxonomically and phylogenetically by the amplified 16S rDNA sequencing method.36) The top 10 organisms with high percentage sequence similarity are shown in the right-hand column of the table. The neighbor-joining tree is shown in Fig. 1. The results indicate that the best overall match was with the genus Sporosarcina. This genus consists of a recently emended large cluster37) including former Bacillus species that belong to rRNA group 2 and some non-Bacillus-type organisms.38) However, identification to the species level was not possible owing to certain features in the phylogenetic tree, as well as insufficiently high percentage similarity of less than 98%. The Sporosarcina strains might be classified into three or four phylogenetic groups, and strain eSP04 appeared to belong to a different group than most of the displayed species or Sporosarcina sp. 5-4. It is concluded that strain eSP04 producing EstAC is a strain of Sporosarcina species. We designated it Sporosarcina sp. nov. strain eSP04.

Purification and molecular weight of the enzyme

EstAC was purified 780-fold from crude cell extracts of strain eSP04 to a specific activity of 150 U/mg of protein at a yield of 7.1% by a 5-step procedure of salting-out and column chromatography (Table 2). The homogeneity of the purified enzyme was examined by SDS–PAGE under reducing conditions (Fig. 2A). The purified enzyme gave a single protein band correspond-
ing to a molecular weight of approximately 43,000. Gel filtration indicated a molecular weight of 48,000/C6/C6; under non-denaturing conditions (Fig. 2B), suggesting EstAC should be present as a monomer.

Since the amount of purified enzyme obtained was 0.12 mg with 7.1% recovery, the content of the enzyme in the cells of strain eSP04 was assessed to be at most 0.13% of total soluble proteins. This low level of the enzyme in the cells would make it difficult to study the essential enzymatic properties of EstAC. The cloning of the esterase gene from Sporosarcina sp. nov. and its overexpression is one attractive way to overcome this problem. As the first step of such an approach, we determined an internal sequence as well as the N-terminal amino acid sequence from the purified enzyme by Edman degradation, as described below.

**Characterization of the enzyme**

Partially purified enzyme instead of purified enzyme was used for activity measurements for the most part (see "Materials and Methods"). Partially purified EstAC from the DEAE-5PW column always showed just half the activity of the purified enzyme. For instance, the former enzyme preparation gave a specific activity of 73 ± 5 U/mg for EBz at pH 8.0 and 35°C, whereas the latter gave 150 ± 8 U/mg under the same experimental conditions. The use of the partially purified enzyme was not thought to have any adverse effects in this study, although a faint band at around 60,000 was associated with this enzyme preparation (Fig. 2A), the origin of which was not clear.

The benzoyl esterase activity of EstAC displayed an optimum pH at around 9 (Fig. 3A), an unexpectedly alkaline pH for carboxylesterase. Protein electrostatics investigation suggested that the active site of esterases and lipases displayed a negative potential in the pH range associated with their maximum activity, and that the esterases showed their optimum charge at pH 6 to 7, which correlated with their usually lower pH-activity optimum than that of the lipases around pH 8.1,6) These investigations were carried out using esterases and lipases of which the 3D-structures and amino acid sequences had been experimentally determined. All the enzymes used in these studies had the catalytic triad of S/H/D (or E) and a conserved sequence motif of GXSXG around the active site serine residue. Hence, the observed maximum activity at around pH 9 suggests that EstAC had a catalytic site different from S/H/D. After the enzyme was maintained without substrate at pH 4.0–10.0 and 4°C for 50 h, more than 65% activity was retained, and more than 90% was retained between pH 8.0 and 10.0 (Fig. 3C). Thus EstAC can be said to have been fairly stable over a wide range of pH. The temperature optimum was between 40°C and 45°C, with a rapid decrease in activity at higher temperatures (Fig. 3B). EstAC appeared to be somewhat heat-labile: it lost 5% and 45% of its activity at pH 8.0 after 60 min incubation without substrate at 40°C and 45°C respectively (Fig. 3D).

The effects of inhibitors on activity are summarized in Table 3. The activity of EstAC was completely inhibited

### Table 2. Purification of an Aryl-carboxylesterase (EstAC) from Sporosarcina sp. nov. Strain eSP04

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Activity (U)</th>
<th>Yield (%)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1,310</td>
<td>260</td>
<td>100</td>
<td>0.20</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>1,070</td>
<td>240</td>
<td>92</td>
<td>0.22</td>
<td>1.1</td>
</tr>
<tr>
<td>DEAE Sepharose FF</td>
<td>139</td>
<td>196</td>
<td>75</td>
<td>1.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Phenyl-650S</td>
<td>7.30</td>
<td>75.0</td>
<td>29</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>DEAE-5PW</td>
<td>0.46</td>
<td>34.0</td>
<td>13</td>
<td>74</td>
<td>370</td>
</tr>
<tr>
<td>Superdex 75 pg</td>
<td>0.12</td>
<td>18.5</td>
<td>7.1</td>
<td>150</td>
<td>780</td>
</tr>
</tbody>
</table>

*aSteps carried out with 2,000 mL of culture broth.

*bEnzyme activity was measured for ethyl benzoate (EBz) at pH 8.0 and 35°C, as described in "Materials and Methods.”

---

**Fig. 2.** Gel Electrophoresis Analysis of the Purified Fraction of EstAC from Sporosarcina sp. nov. eSP04 and Estimation of the Molecular Weight of the Esterase.

(A) SDS–PAGE analysis of EstAC. Lane 1, crude cell extract; lane 2, DEAE-5PW fraction; lane 3, Superdex 75 pg fraction; M, molecular weight markers (BioRad Laboratories). (B) Gel filtration (Superdex 75 pg) chromatogram the purified EstAC (solid circle) under non-denaturing conditions. Hollow circles, labeled 1 to 4, represent the standard proteins (bovine serum albumin, 67,000; ovalbumin, 43,000; chymotrypsinogen A, 25,000; ribonuclease, 13,700). \( V_e \), elution volume for the protein; \( V_0 \), column void volume; \( V_t \), column total bed volume.
in the presence of 0.1 mM phenylmethylsulfonyl fluoride, suggesting that serine residues were involved in the enzyme activity. Compared with the enzyme concentration, the presence of extremely high concentrations of acetic anhydride, \( p\)-chloromercuribenzoate, 2-mercaptoethanol, EDTA, and \( o\)-phenanthroline did not significantly inhibit the activity, suggesting that amino groups, sulfhydryl groups, disulfide bonds, and metal ions were not significantly involved in it. \( N\)-Bromosuccinimide inhibited the activity more significantly than these five chemicals. Since this particular compound cleaves tryptophanyl and tyrosyl peptide bonds and oxidizes sulfhydryl groups,\(^{40,41}\) this result suggests the involvement of tryptophan or tyrosine residues in the enzyme activity. The presence of detergents, which are denaturing agents, also inhibited the activity to a considerable extent: for example, 0.1% Triton X-100 reduced the activity to 49 ± 5%.

The determined N-terminal and internal amino acid sequences of EstAC were ([M?GGNVEKTKLQETLDQLAN (or R) and IADIMVLGDGDENGKDLRK respectively. The first methionine of the N-terminal might have been digested by a methionine aminopeptidase of \( Sporosarcina \) sp. nov. The comparison of these sequences with those of proteins in the databases revealed that the internal sequence had 58% (11/19) and 53% (10/19) homology with two bacterial esterases, and 58% (11/19) with one putative esterase. The former two esterases are 4-chloro-3-hydroxybutyrate hydrolase from \( Rhizobium \) DS-S-51, showing hydrolytic activity toward \((R)-4\)-chloro-3-hydroxybutyrate,\(^{42}\) and 1,4-butanediol diacrylate esterase from \( Brevibacterium linens \), giving 4-hydroxybutyl acrylate.\(^{43}\) The latter was deduced from the genome of a bacterium, \( Rhodopseudomonas palustris \).\(^{44}\) Aryl-carboxylesterase activity remains undetermined for all these enzymes. The N-terminal sequence did not show significant similarity to any known hydrolases.

Bacterial esterases are classified into eight families according to conserved sequence motifs and biological properties.\(^{45}\) The two esterases described above are classified in family VIII. Esterases in family VIII have the catalytic serine residue in the conserved sequence motif SXXK, which is different from the case of GXSSXG around the nucleophilic serine, and usually show an alkaline pH-activity optimum. In addition, they have also been reported to have a tyrosine residue that can be regarded as acting as the general base\(^{46}\) in a consensus motif of YXXN. The putative esterase also had the same motifs, SXXK and YXXN. Taking this homology in internal amino acid sequence, the alkaline pH-activity optimum, and the effects of the inhibitors into consideration, EstAC might be a member of family VIII esterases.

**Table 3. Effects of Enzyme Inhibitor on the Activity of EstAC**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Phenylmethanesulfonyl fluoride</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>Acetic anhydride</td>
<td>1.0</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>( p)-Chloromercuribenzoate</td>
<td>1.0</td>
<td>84 ± 7</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>1.0</td>
<td>82 ± 5</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>( o)-Phenanthroline</td>
<td>1.0</td>
<td>81 ± 9</td>
</tr>
<tr>
<td>( N)-Bromosuccinimide</td>
<td>1.0</td>
<td>60 ± 7</td>
</tr>
</tbody>
</table>

Enzyme activity was measured for EBz using a Metrohm pH-stat at pH 8.0 and 35°C (see “Materials and Methods”). The total volume, 15 mL, of the reaction mixture initially consisted of 4 mM EBz, 5.5 μg of enzyme, 5% DMSO, and 25 mM Tris–HCl buffer solution besides the inhibitor.

The determined N-terminal and internal amino acid sequences of EstAC were ([M?GGNVEKTKLQETLDQLAN (or R) and IADIMVLGDGDENGKDLRK respectively. The first methionine of the N-terminal might have been digested by a methionine aminopeptidase of \( Sporosarcina \) sp. nov. The comparison of these sequences with those of proteins in the databases revealed that the internal sequence had 58% (11/19) and 53% (10/19) homology with two bacterial esterases, and 58% (11/19) with one putative esterase. The former two esterases are 4-chloro-3-hydroxybutyrate hydrolase from \( Rhizobium \) DS-S-51, showing hydrolytic activity toward \((R)-4\)-chloro-3-hydroxybutyrate,\(^{42}\) and 1,4-butanediol diacrylate esterase from \( Brevibacterium linens \), giving 4-hydroxybutyl acrylate.\(^{43}\) The latter was deduced from the genome of a bacterium, \( Rhodopseudomonas palustris \).\(^{44}\) Aryl-carboxylesterase activity remains undetermined for all these enzymes. The N-terminal sequence did not show significant similarity to any known hydrolases.

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**Substrate specificity**

The specific activities for a wide variety of aryl-carboxylic acid esters are summarized in Table 4. Several of the substrates were also examined with the purified enzyme to compare results. This confirmed the appropriateness of using the partially purified EstAC. The pH and temperature for the specific activity measurements were determined by considering spontaneous hydrolysis, the solubility of the substrates, and the stability of the enzyme. EstAC displayed the highest activity not only for EBz and methyl benzoate, but also for DET, of all the substrates examined. Our examination revealed that among previously reported aryl-carboxylesterases, an esterase from *A. nomius* was found to be an attractive biocatalyst having stereoselectivity of the enzyme indicated that EstAC is specific for the enantiomers of ethyl 2-(4-isobutylphenyl)propionate and ethyl 3-phenylbutanoate respectively. The enantiomer excesses of the two products were 40% and 30% at 20% yield, respectively. This indicates that the enzyme shows the same structure with regard to the bulkiness of substituents.

EstAC also showed high activities for dialkyl esters of dibasic aryI-carboxylic acids and dihydric alcohol esters of aryl-carboxylic acids in addition to ordinary esters of aryl-carboxylic acid, as described above. It catalyzed the hydrolysis of DET, dimethyl terephthalate, dimethyl isophthalate, and EGDB, although it did not hydrolyze phthalates. DET and EGDB appear to have chemical structures that are the inverse of each other. The specific activity for EGDB was 30% of DET and of EBz. Nevertheless, the activity value for EGDB (Table 4) was substantially high and even greater than that of a remarkable benzoyl esterase from *Rhodotorula mucilaginosa*, previously reported. EstAC showed no activity on monomethyl terephthalate. This suggests that *Sporosarcina* sp. strain esP04 has another enzyme to hydrolyze monoaIyl terephthalate, since it grew on DET as sole carbon source and consumed it during the production of the enzyme.

EstAC was highly active on *p*-nitrophenyl acetate, as previously reported for various esterases, including aryl-carboxylesterases. However, it showed a relatively low activity for an ester having a phenoxyl group in an alcohol moiety, such as 1-phenoxyl-2-propyl acetate, which was easily hydrolyzed with various lipases.

This again confirmed that EstAC is specific for the aromatic acid moiety. A preliminary experiment on the enantioselectivity of the enzyme indicated that EstAC preferentially hydrolyzed the (R)- and (S)-enantiomers of ethyl 2-(4-isobutylphenyl)propionate and ethyl 3-phenylbutanoate respectively. The enantiomer excesses of the two products were 40% and 30% at 20% yield, respectively. This indicates that the enzyme prefers the same structure with regard to the bulkiness of substituents.

**Substrate Specificity of an Aryl-carboxylesterase EstAC**

*Table 4. Substrate Specificity of an Aryl-carboxylesterase EstAC*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>Specific activity purified enzyme (U/mg)</th>
<th>Specific activity partially purified enzyme (U/mg)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Butyl benzoate</td>
<td>4</td>
<td>63 ± 7</td>
<td>63 ± 7</td>
<td>86 ± 10</td>
</tr>
<tr>
<td>Ethyl benzoate</td>
<td>4</td>
<td>150 ± 8</td>
<td>73 ± 5</td>
<td>100</td>
</tr>
<tr>
<td>Methyl benzoate</td>
<td>4</td>
<td>83 ± 16</td>
<td>113 ± 20</td>
<td></td>
</tr>
<tr>
<td>Ethyl phenylacetate</td>
<td>4</td>
<td>43 ± 10</td>
<td>18 ± 5</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>Methyl phenylacetate</td>
<td>4</td>
<td>24 ± 3</td>
<td>33 ± 4</td>
<td></td>
</tr>
<tr>
<td>(+)-Ethyl mandelate</td>
<td>4</td>
<td>45 ± 9</td>
<td>16 ± 4</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>(+)-Ethyl 2-(4-isobutylphenyl)propionate</td>
<td>4</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>(+)-Ethyl 2-methyl-3-phenylpropionate</td>
<td>4</td>
<td>22 ± 5</td>
<td>30 ± 7</td>
<td></td>
</tr>
<tr>
<td>(+)-Ethyl 3-phenylbutanoate</td>
<td>4</td>
<td>40 ± 1.6</td>
<td>5.4 ± 2</td>
<td></td>
</tr>
<tr>
<td>Dethyl terephthalate</td>
<td>2</td>
<td>160 ± 13</td>
<td>74 ± 3</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>Dimethyl terephthalate</td>
<td>2</td>
<td>51 ± 2</td>
<td>69 ± 3</td>
<td></td>
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<tr>
<td>Monomethyl terephthalate</td>
<td>2</td>
<td>~0</td>
<td>24 ± 2</td>
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<tr>
<td>Dimethyl isophthalate</td>
<td>2</td>
<td>35 ± 7</td>
<td>~0</td>
<td>0</td>
</tr>
<tr>
<td>Dethyl phthalate</td>
<td>2</td>
<td>~0</td>
<td>~0</td>
<td>0</td>
</tr>
<tr>
<td>Dimethyl phthalate</td>
<td>2</td>
<td>~0</td>
<td>~0</td>
<td>0</td>
</tr>
<tr>
<td>Ethylene glycol dibenzoate</td>
<td>2</td>
<td>22 ± 4</td>
<td>30 ± 5</td>
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</tr>
<tr>
<td>Tributyrin</td>
<td>4</td>
<td>13 ± 5</td>
<td>6.0 ± 3</td>
<td>8.1 ± 4</td>
</tr>
<tr>
<td>1-Phenoxy-2-propyl acetate</td>
<td>4</td>
<td>1.8 ± 0.7</td>
<td>2.5 ± 0.9</td>
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</tr>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>4</td>
<td>45 ± 13</td>
<td>62 ± 18</td>
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</tr>
</tbody>
</table>

Specific activity was measured at pH 8.0 and 35 °C using a Metrohm pH-stat system. The total volume, 15 mL, of the reaction mixture initially consisted of 6.7–22.0 μg of partially purified enzyme or 2.7–8.0 μg of purified enzyme, 5% DMSO, and 25 mM Tris–HCl buffer solution besides the substrate.

*Relative activity is expressed as a percentage of the specific activity obtained with ethyl benzoate.

*The substrate was insoluble under the experimental conditions. The reaction mixture was emulsified by vigorous stirring (see "Materials and Methods").*
enzyme. Purified recombinant EstAC hydrolyzed ethyl 4-hydroxybenzoate and ethyl cinamamate with 1.2% and 65% activities of EBz respectively (unpublished results, our laboratory). This indicates that the enzyme has 4-hydroxybenzoyl and cinamoyl esterase activities as well. The former activity might be regarded as being too low for practical use, but it is much higher than that of a recently reported new 4-hydroxybenzoyl esterase from *Aspergillus oryzae*, although the experimental conditions were slightly different. 18)

### Acknowledgment

We thank Professor Shuichi Karita of Mie University for his help with N-terminal amino acid sequence analysis.

### References