Biochemical and Molecular Characterization of a Ring Fission Dioxygenase with the Ability to Oxidize (Substituted) Salicylate(s) from *Pseudaminobacter salicylatoxidans*

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The gene coding for a dioxygenase with the ability to cleave salicylate by a direct ring fission mechanism to 2-oxohepta-3,5-dienedioic acid was cloned from *Pseudaminobacter salicylatoxidans* strain BN12. The deduced amino acid sequence encoded a protein with a molecular mass of 41,176 Da, which showed 28 and 31% sequence identity, respectively, to a gentisate 1,2-dioxygenase from *Pseudomonas alcaligenes* NCIMB 9867 and a 1-hydroxy-2-naphthoate 1,2-dioxygenase from *Nocardioides* sp. KP7. The highest degree of sequence identity (58%) was found to a presumed gentisate 1,2-dioxygenase from *Corynebacterium glutamicum*. The enzyme from *P. salicylatoxidans* BN12 was heterologously expressed in *Escherichia coli* and purified as a His-tagged enzyme variant. The purified enzyme oxidized in addition to salicylate, gentisate, 5-aminosalicylate, and 1-hydroxy-2-naphthoate also 3-amino- and 3- and 4-hydroxysalicylate, 5-fluorosalicylate, 3-, 4-, and 5-chlorosalicylate, 3-, 4-, and 5-bromosalicylate, 3-, 4-, and 5-methylsalicylate, and 3,5-dichlorosalicylate. The reactions were analyzed by high pressure liquid chromatography/mass spectrometry, and the reaction products were tentatively identified. For comparison, the putative gentisate 1,2-dioxygenase from *C. glutamicum* was functionally expressed in *E. coli* and shown to convert gentisate but not salicylate or 1-hydroxy-2-naphthoate.

The oxygenolytic cleavage of the aromatic nucleus by bacteria requires in most cases the presence of two hydroxy groups attached to the aromatic ring (1–3). Only a few examples have been described previously in which monohydroxylated aromatic compounds were cleaved by ring fission dioxygenases, and in most of these examples aminohydroxybenzene derivatives were observed as ring fission substrates. The ability of “traditional” ring fission dioxygenases to oxidize aminohydroxybenzene derivatives is mechanistically easily explained, because the amino group activates the aromatic nucleus in a similar way as the hydroxy group for an electron-donating substituent. This has been described for the oxidation of 5-chlorosalicylate by a “Bacillus” sp. and for the conversion of 1-hydroxy-2-naphthoate by several Gram-negative (e.g. *Aeromonas* sp.) and Gram-positive bacteria (*Nocardioides* sp.) From these two reactions only the oxidation of 1-hydroxy-2-naphthoate by *Nocardioides* sp. KP7 has been analyzed on an enzymatic and genetic level, and the ring fission product was isolated and characterized by various spectrosopic techniques (10–14).

We recently described a new ring fission dioxygenase from the naphthalenesulfonate-degrading strain *Pseudaminobacter salicylatoxidans*, which oxidized salicylate by a novel ring fission mechanism to 2-oxohepta-3,5-dienedioic acid (Scheme 1). The ring fission dioxygenase resembled gentisate 1,2-dioxygenases or 1-hydroxy-2-naphthoate dioxygenases, because of the ability of the enzyme to convert gentisate and 1-hydroxy-2-naphthoate, the size of the subunits, the structure of the holoenzyme, and the dependence of the enzyme on Fe^{2+} ions (15). In order to allow a more detailed analysis of the ability of ring fission dioxygenases to oxidatively cleave the aromatic ring of monohydroxylated benzene derivatives in the current study, the encoding gene was cloned and the substrate specificity of the enzyme analyzed in greater detail.

**MATERIALS AND METHODS**

**Bacterial Strains and Media—**The isolation and characterization of *P. salicylatoxidans* strain BN12 DSM 6986^{T} has been described previously (16, 17). *Corynebacterium glutamicum* ATCC 13032 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *Escherichia coli* JM109 and *E. coli* BL21(DE3) were used as host strains for recombinant DNA work.

Mineral media were prepared as described by Dorn et al. (18) and were supplemented with 100 mg/ml of yeast extract. *P. salicylatoxidans* was routinely grown in this supplemented mineral medium with 5 mM 6-aminonaphthalene-2-sulfonate. For the cultivation of *C. glutamicum*, a complex medium proposed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen was used. The recombinant *E. coli* strains were routinely cultured in Luria-Bertani medium supplemented with ampicillin (100 μg/ml).

Heat-labile and autoxidable substrates were sterilized by membrane filtration (pore size, 0.2 μm; Sartorius, Göttingen, Germany); all other substrates were autoclaved at 121 °C.

**Oxygen Uptake Experiments—** *P. salicylatoxidans* BN12 was grown in a mineral medium with 6-aminonaphthalene-2-sulfonate (5 mM) supplemented with 100 mg/ml yeast extract. The cells were harvested during the late exponential growth phase by centrifugation and resuspended in Tris-HCl buffer (20 mM, pH 8) to an optical density (A_{600nm}) of 14. The resting cells were incubated at 30 °C in an oxygen electrode (YSI 5350, YSI Inc., Yellow Springs, OH). The endogenous respiration was determined for 2 min, and then the respective salicylates were added (1 mM each). The oxygen uptake was recorded for 5 min, and the reaction rates were corrected for the endogenous respiration.

**Preparation of Cell Extracts—**Cell suspensions in 20 mM Tris-HCl buffer (pH 8.0) were disrupted by using a French press (Aminco, Silver Springs, MD) at 80 MPa. Cell debris was removed by centrifugation at 3000 × g for 10 min.

Received for publication, December 10, 2003, and in revised form, June 17, 2004. Published, JBC Papers in Press, June 25, 2004, DOI 10.1074/jbc.M313500200

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AT323951.
Micromass, Manchester, UK) using electrospray ionization in the negative mode. The separated compounds from HPLC were subsequently analyzed by LC–MS (using a MS Manager 2.0, Waters, Milford, MA). A reversed-phase column (Lichrospher RP8 (end capped) was used. The concentrations of the substrates were determined by Bradford (19) with bovine serum albumin as a standard.

Protein Purification, Enzymatic Cleavage of the Protein, Isolation of Peptides, and Sequencing of Peptides and Amino Termini—The dioxygenase was purified from P. salicylatoxidans NCIB 9262 by fast protein liquid chromatography as described previously (15). The digestion of the purified dioxygenase by trypsin and the subsequent separation of tryptic digests by reversed-phase HPLC were performed by established procedures (20). The amino acid sequences were determined by automated Edman degradation by using an ABI 476 protein sequence analyzer (Applied Biosystems, Foster City, CA). PAGE–SDS-PAGE was performed by the method of Laemmli (21), and the gels were routinely stained with Coomassie Blue.

Enzyme Assays with Purified Enzyme Preparations—One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate/min. The conversion of 1-hydroxy-2-naphthoate, gentisate, 5-aminosalicylate, and salicylate were determined spectrophotometrically as described previously (15). For the determination of the kinetic parameters, the kinetic parameters were determined basically as described above but using a substrate concentration range from 0.01 to 5 mM. The enzyme reaction was monitored spectrophotometrically at 210 nm and at the wavelength indicated in Table 1 by using a photodiode array detector.

Analytical Methods—The turnover of the purified enzyme was analyzed by reversed-phase HPLC (HPLC pumps model 510 equipped with a photo-diode array detector model 996 and Millenium Chromatography Manager 2.0, Waters, Milford, MA). A reversed-phase column (150 × 4.0 mm, internal diameter) packed with 5-μm particles of Merck Lichrospher RP8 (end capped) was used. The separated compounds were detected photometrically at 210 nm and at the wavelength indicated in Table 1 by using a photodiode array detector.

Liquid Chromatography-Mass Spectrometry—Product identification was performed by liquid chromatography-mass spectrometry (HP1100, Agilent) coupled to a triple quadrupole mass spectrometer (Quattro LC, Micromass, Manchester, UK) using electrospray ionization in the negative ion mode. Substrate solutions before and after addition of the enzyme and 20 min after addition of the enzyme were injected (20 μl) into the HPLC system without any pretreatment. Analytes were separated by ion-pair chromatography on a Luna C18 (2) 3-μm column, 15 cm × 3 mm inner diameter at 40 °C. Eluent A was H2O/MeOH (60:40 v/v), and eluent B was H2O/MeOH (5:95 v/v) with 1 μM tributylamine and 1 μM acetic acid each. Gradient elution started with 20% (v/v) eluent B at 0 min, 90% eluent B at 11 min, isocratic to 15 min, and 16 min 20% (v/v) eluent B, 21 min, 20% (v/v) eluent B. A diode array detector and the MS were coupled in a series. The mass spectrometric interface was operated at a cone voltage of 18 V and a capillary voltage of 2.9 kV. Probe temperature was 220 °C, and source block temperature was 120°C. Product ion spectra were recorded at collision energies of 10 and 15 eV with a scan rate of 0.5 s.

PCR—Oligonucleotides were custom-synthesized according to the known or deduced sequences of the amino-terminal amino acid sequence and various internal peptides of the ring fission dioxygenase.

For the amplification reaction with the primers deduced from the amino terminus of the protein, the primers NcoI-fwd 5’-CCGGATCCTGATTCCG-3’ and NcoI-rev 5’-CTTCTGGTCTGC-3’ were used. For the amplification reactions the following PCR programs were used: an initial denaturation (94 °C, 3 min) was followed by the cycles consisting of an annealing temperature of 38.7 °C (30 s), a polymerization step (72 °C, 2 min), and denaturation (94 °C, 30 s). The last polymerization step was extended to 15 min.

For the determination of the complete sequence of the gene encoding the C. glutamicum 1,2-dioxygenase, a PCR product was obtained from the previously cloned NdeI fragment by trypsin digestion and subsequent separation of the tryptic digests by reversed phase HPLC. The amino acid sequences were determined by automated Edman degradation by using an ABI 476 protein sequence analyzer (Applied Biosystems, Foster City, CA). PAGE–SDS-PAGE was performed by the method of Laemmli (21), and the gels were routinely stained with Coomassie Blue.

Enzyme Assays with Purified Enzyme Preparations—One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate/min. The enzyme reactions were monitored spectrophotometrically at 210 nm and at the wavelength indicated in Table 1 by using a photodiode array detector.

Analytical Methods—The turnover of the substituted salicylates was analyzed by reversed-phase HPLC (HPLC pumps model 510 equipped with a photo-diode array detector model 996 and Millenium Chromatography Manager 2.0, Waters, Milford, MA). A reversed-phase column (150 × 4.0 mm, internal diameter) packed with 5-μm particles of Merck Lichrospher RP8 (end capped) was used. The separated compounds were detected photometrically at 210 nm and at the wavelength indicated in Table 1 by using a photodiode array detector.

Liquid Chromatography-Mass Spectrometry—Product identification was performed by liquid chromatography-mass spectrometry (HP1100, Agilent) coupled to a triple quadrupole mass spectrometer (Quattro LC, Micromass, Manchester, UK) using electrospray ionization in the negative ion mode. Substrate solutions before and after addition of the enzyme and 20 min after addition of the enzyme were injected (20 μl) into the HPLC system without any pretreatment. Analytes were separated by ion-pair chromatography on a Luna C18 (2) 3-μm column, 15 cm × 3 mm inner diameter at 40 °C. Eluent A was H2O/MeOH (60:40 v/v), and eluent B was H2O/MeOH (5:95 v/v) with 1 μM tributylamine and 1 μM acetic acid each. Gradient elution started with 20% (v/v) eluent B at 0 min, 90% eluent B at 11 min, isocratic to 15 min, and 16 min 20% (v/v) eluent B, 21 min, 20% (v/v) eluent B. A diode array detector and the MS were coupled in a series. The mass spectrometric interface was operated at a cone voltage of 18 V and a capillary voltage of 2.9 kV. Probe temperature was 220 °C, and source block temperature was 120°C. Product ion spectra were recorded at collision energies of 10 and 15 eV with a scan rate of 0.5 s.

PCR—Oligonucleotides were custom-synthesized according to the known or deduced sequences of the amino-terminal amino acid sequence and various internal peptides of the ring fission dioxygenase.

For the amplification reaction with the primers deduced from the amino terminus of the protein, the primers NcoI-fwd 5’-CCGGATCCTGATTCCG-3’ and NcoI-rev 5’-CTTCTGGTCTGC-3’ were used. For the amplification reactions the following PCR programs were used: an initial denaturation (94 °C, 3 min) was followed by the cycles consisting of an annealing temperature of 55 °C (30 s), a polymerization step (72 °C, 2.5 min), and denaturation (94 °C, 30 s). The last polymerization step was extended to 15 min. The PCR products were initially cloned into the T-tailed EcoRV-site of pBluescript II KS(+) (23).

Expression of the Dioxygenase in E. coli—For expression in E. coli, the dioxygenase gene was amplified from the plasmid vector pET28a (Novagen, Madison, WI) under the control of the T7 promoter. The DNA segment encompassing the dioxygenase gene was amplified by PCR (using a Pwo DNA-polymerase; Peqlab). The upstream primer (5’-GAGGTCGATATGTCAGAACG-3’) incorporated an NdeI site (underlined), and the downstream primer (5’-CGGATCTCATGCATCGCCTCGG-3’ incorporated a BamHI site (underlined). The amplified product was cloned into the EcoRV site of pBluescript II KS(+), The resulting plasmid was cleaved with NdeI and BamHI and the DNA fragment with the dioxygenase gene ligated into pET28a, which was also previously cut with NdeI and BamHI. The resulting plasmid (pJPH100exN) was subsequently transformed into E. coli BL21(DE3).

Expression of the dioxygenase gene was induced by 1-thio-β-β-galactopyranoside as suggested by the supplier of the pET system.

Purification of the His-tagged Enzyme—Cell extracts of E. coli JM109(pJPH100exN) were prepared in Tris-HCl buffer (20 mM, pH 8) as described above. The nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany) was suspended in Tris-HCl (20 mM, pH 8) and transferred (2 ml) to an empty 10-ml polypropylene column. The filled column was equilibrated with a buffer system (pH 8) consisting of Tris-HCl (20 mM), NaCl (300 mM), and imidazole (50 mM). The cell extracts (about 100 mg of protein) were applied to the column, and the protein was eluted with subsequent steps of the Tris-HCl/NaCl buffer (2–4 ml each) consisting of increasing imidazole concentrations (50–500 mM). The fractions with dioxygenase activity eluted at an imidazole concentration of 150 mM. The imidazole was removed from the active fractions on a “HiTrap desalting column” (Amersham Biosciences) using Tris-HCl (20 mM, pH 8) plus 100 mM NaCl as eluent buffer.

Cloning of the Presumed Gentisate 1,2-Dioxygenase from C. glutamicum—The gene was amplified from the genomic DNA by using the oligonucleotide primers GDO-Cglu-fwd 5’-CAGCATATGGGCGCCCGAG-3’ and GDO-Cglu-rev 5’-CAGATTCTGATGGGG-3’. These primers were designed from the deduced amino acid sequence of the amino-terminal amino acid sequence and various internal peptides of the ring fission dioxygenase.

The abbreviations used are: HPLC, high pressure liquid chromatography; MS, mass spectrometry; LC-MS, liquid chromatography-mass spectrometry.
several peptides were isolated by HPLC. The amino acid sequence was determined by dideoxy chain termination with double-stranded DNA of clones and overlapping subclones in an automated DNA sequencing system (ALF Sequencer, Amersham Biosciences) with fluorescently labeled primers or nucleotides. Sequence analysis, data base searches, and comparisons were done using the NCBI facilities. The alignment of the ring fission dioxygenase was unequivocally identified in this sequence by the presence of the amino-terminal region and the internal peptides determined before by Edman degradation (Fig. 1).

**Results**

**Oxidation of Different Salicylates by Resting Cells of P. salicylatoxidans BN12**—It was demonstrated previously that the enzyme from *P. salicylatoxidans* BN12 oxidized salicylate, gentisate, 5-aminosalicylic acid, and 1-hydroxy-2-naphthoate by an 1,2-dioxygenolytic cleavage (15). This ring fission mechanism principally allows the cleavage of various substituted salicylates. Therefore, resting cells of *P. salicylatoxidans* BN12 were incubated with a wide range of substituted salicylates, and the substrate-dependent oxygen uptake rates were determined. These experiments suggested that the bacteria were also able to oxidize a wide range of methyl-, chloro-, or bromo-substituted salicylates. Most surprisingly, the cells oxidized 5-fluorosalicylic acid with its exceptionally electronegative fluorine substituent with higher rates than salicylate (Fig. 1).

**Cloning of the Gene Encoding the Salicylate 1,2-Dioxygenase**—The ring fission dioxygenase was purified from cell extracts of *P. salicylatoxidans* BN12 as described previously (15). The purified dioxygenase was digested with trypsin, and several peptides were isolated by HPLC. The amino acid sequences of the amino terminus and five internal fragments were determined and used for the design of oligonucleotide primers for PCR experiments (Table II). Using genomic DNA of *P. salicylatoxidans* BN12 as template and primers derived from the amino-terminal sequence and of the peptide P28 (Table II), a DNA fragment with a size of about 0.7 kb was amplified. The amplified fragment was sequenced, and the sequence obtained was used to complete the sequence of the gene by using partial inverse PCR (see “Material and Methods”). Thus a continuous stretch of DNA of 1249 bp was obtained. The gene for the salicylate dioxygenase was unequivocally identified in this sequence by the presence of the amino-terminal region and the internal peptides determined before by Edman degradation (Fig. 1).

**Expression of the Dioxygenase Gene in E. coli**—The dioxygenase gene was amplified by PCR from the genomic DNA of strain BN12 by using a set of primers that created new NdeI and BamHI restriction sites. The amplified fragment was then ligated into plasmid pBluescript II SK+ (previously cut with

| Solvent system (% methanol) | Solvent system (% methanol) | Substrate | Substrate | (Main) product | (Main) product | Absorption maximum | Absorption maximum | Absorption maximum | Absorption maximum |
|-----------------------------|-----------------------------|----------|----------|---------------|---------------|--------------------|--------------------|--------------------|--------------------|
| 3-Aminosalicylic acid       | 10                          | 3.0      | 300      | ND            | ND            | 37252              | 1.7                | 274                |
| 4-Aminosalicylic acid       | 10                          | 7.6      | 300      | ND            | ND            | 37252              | 1.7                | 274                |
| 5-Aminosalicylic acid       | 40                          | 4.8      | 3117     | ND            | ND            | 37252              | 1.7                | 274                |
| 3-Hydroxysalicylic acid     | 40                          | 4.8      | 317      | 2.3           | 332           | 37252              | 1.6                | 246                |
| 4-Hydroxysalicylic acid     | 40                          | 4.7      | 295      | 1.6           | 246           | 37252              | 1.7                | 210                |
| 6-Hydroxysalicylic acid     | 40                          | 3.6      | 308      | NR            | NR            | 37252              | 1.7                | 210                |
| 3-Methylsalicylic acid      | 60                          | 3.3      | 309      | 1.7           | 210           | 37252              | 1.7                | 217                |
| 4-Methylsalicylic acid      | 60                          | 5.1      | 302      | 1.7           | 210           | 37252              | 1.7                | 217                |
| 5-Methylsalicylic acid      | 60                          | 5.1      | 315      | 1.7           | 205           | 37252              | 1.7                | 205                |
| 5-Fluorosalicylic acid      | 60                          | 4.4      | 313      | 1.6           | 293           | 37252              | 1.8                | 217                |
| 3-Chlorosalicylic acid      | 60                          | 5.3      | 309      | 1.8           | 217           | 37252              | 1.8                | 217                |
| 4-Chlorosalicylic acid      | 60                          | 7.7      | 302      | 1.7           | 217           | 37252              | 1.7                | 217                |
| 5-Chlorosalicylic acid      | 60                          | 6.9      | 315      | 1.8           | 300           | 37252              | 1.8                | 300                |
| 3-Bromosalicylic acid       | 60                          | 6.1      | 309      | 1.8           | 223           | 37252              | 1.8                | 223                |
| 4-Bromosalicylic acid       | 60                          | 8.9      | 302      | 1.8           | 222           | 37252              | 1.8                | 300                |
| 5-Bromosalicylic acid       | 60                          | 9.0      | 315      | 1.8           | 300           | 37252              | 1.8                | 300                |
| 5-Iodosalicylic acid        | 60                          | 9.4      | 320      | NR            | NR            | 37252              | 1.8                | 300                |
| 5-Nitrosalicylic acid       | 60                          | 3.6      | 308      | NR            | NR            | 37252              | 1.8                | 300                |
| 5-Sulfosalicylic acid       | 60                          | 4.1      | 302      | NR            | NR            | 37252              | 1.8                | 300                |
| 3,5-Dichlorosalicylic acid  | 60                          | 10.8     | 319      | 1.6           | 210           | 37252              | 1.7                | 200                |
| 3,5-Dibromosalicylic acid   | 60                          | 14.3     | 319      | 1.7           | 200           | 37252              | 1.7                | 200                |
| 3,5-Diodosalicylic acid     | 60                          | 21.5     | 328      | NR            | NR            | 37252              | 1.7                | 200                |
| Salicylic acid              | 15                          | 22.1     | 300      | 3.3           | 283           | 37252              | 3.3                | 283                |

* ND, not detected.
  NR, no reaction observed.

The solvent systems contained the indicated concentrations of methanol in an aqueous system containing 0.3% (v/v) H3PO4. The usual flow rate was 0.7 ml/min.

DNA Sequencing and Nucleotide Sequence Analysis—The DNA sequence was determined by dideoxy chain termination with double-stranded DNA of clones and overlapping subclones in an automated DNA sequencing system (ALFexpress-Sequence, Amersham Biosciences) with fluorescently labeled primers or nucleotides. Sequence analysis, data base searches, and comparisons were done using the program Clustal using the default parameters.

Chemicals—The chemicals used were obtained from Aldrich, Fluka (Neu-Ulm, Germany), Merck, Serva (Heidelberg, Germany), and Sigma. 6-Aminonaphthalene-2-sulfonate was kindly provided by Bayer AG (Leverkusen, Germany). The complex media were purchased from Difco (Detroit, USA) and Oxoid (Wesel). The suppliers of the substituted salicylates have been described previously (26). The reagents for molecular biology were supplied by Invitrogen, Peqlab (Erlangen, Germany), MBI Fermentas (St. Leon-Rot, Germany), New England Biolabs (Schwalbach, Germany), and Roche Applied Science.
Salicylate 1,2-Dioxygenase from *P. salicylatoxidans*  

The dioxygenase activity of these cell extracts with salicylate as substrate was 0.43 units mg⁻¹ of protein. This was about 25-fold higher compared with the activity found in the wild-type strain of *P. salicylatoxidans* BN12. The His-tagged dioxygenase was purified by affinity chromatography using nickel-nitriolotriacetic acid-agarose (see "Materials and Methods"). This resulted in an enzyme preparation that was more than 95% homogenous, according to SDS-PAGE, and that converted salicylate as substrate with a specific activity of about 2 units/mg protein.

**Spectrophotometric Analysis of the Substrate Conversion by the Purified Dioxygenase**—The oxygen uptake experiments with resting cells of *P. salicylatoxidans* (see above) suggested a broad substrate specificity of the salicylate 1,2-dioxygenase activity. It was demonstrated previously that the conversion of salicylate, 5-amino-, and 5-hydroxy salicylate and also 1-hydroxy-2-naphthoate could be analyzed spectrophotometrically because of the pronounced UV-visible spectra of the products formed (15). Therefore, the reactions of other salicylates were also analyzed by UV-visible spectroscopy. Thus it was found that cell extracts of the recombinant *E. coli* strain converted 3-, 4-, and 5-substituted amino-, hydroxy-, chloro-, bromo-, and methylsalicylates (Fig. 3). The conversion of all isomers of the chloro- and bromosalicylates (and also of 5-fluorosalicylate which was the only available fluorosalicylate) resulted in the formation of products with absorption maxima in the range of λ_max = 290–302 nm. The turnover of the methylsalicylates also resulted in the initial formation of new absorbance maxima in the range from 293 to 300 nm. The changes in the spectra observed during the oxidation of the chloro-, bromo-, and methylsalicylates clearly resembled the changes in the UV-visible spectrum determined previously for the formation of 2-oxohepta-3,5-dienedioic acid (λ_max = 283 nm), the product formed from salicylate by the same enzyme (15). The observed bathochromic shifts for the methylated and halogenated derivatives of 2-oxohepta-3,5-dienedioic acid corresponded to the established Woodward diene rules (28).

The changes of the UV-visible spectra observed during the conversion of the isomeric amino- and hydroxysalicylates were different from those observed with the methylated and halogenated salicylates (Fig. 3) but also were consistent with a 1,2-dioxygenolytic cleavage of these substrates. Thus, the turnover of gentisate and 5-aminosalicylate resulted in the formation of new absorption maxima at λ_max = 335 and 352 nm, respectively. These changes in the UV-visible spectra had been described previously for the 1,2-dioxygenolytic cleavage of these two substrates (5, 8, 11). The products formed from 4-hydroxy- and 4-aminosalicylate demonstrated similar absorption maxima at λ_max = 336 and 344 nm.

The oxidation of 3-hydroxysalicylate resulted in the formation of a pronounced new absorption maximum at λ_max = 393 nm. The extraordinary bathochromic shift observed in this reaction could possibly be explained by the almost symmetrical structure of the expected product (2-oxo-6-hydroxyhepta-3,5-dienedioic acid) that allowed keto-enol tautomerizations involving both hydroxy/oxo groups in the 2- and 6-positions. In contrast, with 3-aminosalicylate there was only a decrease in the absorbance at wavelengths >300 nm, and a new absorbance maximum was formed at λ_max = 270 nm (Fig. 3). These spectral changes were presumably due to the ability of the ring fission product of 3-aminosalicylate to undergo an intramolecular cyclization and rearomatization to a pyridinecarboxylic acid shown previously for the reaction products formed by ring fission dioxygenases from 3-hydroxyanthranilate and 2-aminophenol (29, 30). Thus, the results suggested that all substituted salicylates were cleaved by the "salicylate dioxygenase" by the same 1,2-dioxygenolytic mechanism that had been proven previously for the cleavage of salicylate by the same enzyme.

**Analysis of the Enzymatic Reactions by HPLC**—The turnover of various salicylates (0.1 mM each) by the purified (His-tagged) enzyme was also analyzed by HPLC. The results demonstrated that the purified enzyme was indeed able to convert various salicylates that carried methyl-, fluoro-, chloro-, bromo-, or iodo-substituents in the 3-, 4-, or 5-position, although in general with much lower specific activities than observed with 5-amino- or 5-hydroxysalicylate (Table III). No turnover was observed with 6-hydroxysalicylate, 5-carboxysalicylate, 5-sulfosalicylate, 5-nitrosalicylate, 3,5-dibromosalicylate, 3,5-diodosalicylate, or 3,4-dihydroxysalicylate.

The results confirmed that all available 5-substituted salicylates those that carried substituents with electron-donating properties (such as the OH or NH₂ groups) were by far the best substrates. Most surprisingly, 5-substituted salicylates with a weak (such as the methyl group) or very strong (fluorine) elec-
tron-withdrawing effect were also converted with a higher reaction rate than found with the unsubstituted salicylate as a substrate. In contrast to 5-fluorosalicylate, 5-chloro, 5-bromo-, and 5-iodosalicylate were converted with lower reaction rates than salicylate. This might indicate some steric hindrance with these substrates. A similar effect was also found with the 3,5-disubstituted salicylates tested because the enzyme oxidized 3,5-dichlorosalicylate but not 3,5-dibromo- and 3,5-diiodosalicylate.

A comparison of the reaction rates observed with those salicylates for which the respective 3-, 4-, and 5-substituted isomers were available (amino-, hydroxy-, methyl-, bromo-, and chlorosalicylates) suggested that for the salicylates with an activating effect (amino- and hydroxysalicylates), only a substituent in the 5-position resulted in a pronounced increase (\( \times 100 \)) times) in the oxidation rates compared with the respective 3- or 4-substituted isomers. In contrast, in the case of the chloro- or bromo-substituted salicylates, the 5-substituted salicylates were converted with a lower reaction rate than the respective 3- or 4-substituted substrates.

In a further set of experiments it was attempted to determine the \( K_m \) values of the enzyme for different salicylates. The comparability of these experiments was hampered by different

| Table II: Sequences of the amino terminus, tryptic peptides, and deduced oligonucleotides |
|-----------------------------------------------|
| | Protein or peptide | Amino acid sequence | Deduced oligonucleotide sequences |
|-----------------------------------------------|
| Amino terminus | MQNEKLDHESVTQAMQPDKTPNELRALYKS | 5'-CA/AG/CNATGCA/AGCC3' |
| P28 | WEFTDR | 5'-AG/TCNGT/AG/AA/TC/TCCCA3' |
| P32 | WSTLLR | |
| P36 | ALTEQLLEDGEQPAVTAPVGAAR | |
| P52 | ALGPANPGLGNAYTSPMT | |

![Fig. 2. DNA sequence and amino acid sequence of the salicylate dioxygenase from P. salicylatoxidans BN12. The amino-terminal and the internal amino acid sequences, which were determined by chemical Edman degradation, are underlined. The stop codon is indicated by a dash.](image-url)
FIG. 3. Conversion of various substituted salicylates by cell extracts from *E. coli* JM109 (pJPH100exN). The reaction mixtures contained in 1 ml of 20 mM Tris-HCl (pH 8.0), 0.1 mM of the respective salicylate and 1–140 µg of protein. The spectra were recorded every minute against a reference cuvette, which contained the same amount of protein in Tris-HCl buffer but in which the respective substrates were omitted.
degrees of substrate inhibition effects observed with the different substrates but basically confirmed the results obtained with fixed substrate concentrations (Table IV). These experiments demonstrated that the enzyme showed significantly higher $K_{m}$ values with 3- and 5-aminosalicylate compared with other substituted salicylates.

### Analysis of the Enzymatic Reactions by LC-MS/MS—The turnover of several substituted salicylates was also analyzed by LC-MS techniques in order to confirm further the proposed reaction mechanism. In these experiments the reaction mixtures were directly injected into the liquid chromatography system (without terminating the reactions by acidification) and were also analyzed in an almost neutral solvent system (at pH 6.5). Based on LC-MS analyses, two groups of salicylates could be distinguished as follows: (a) compounds showing the expected dioxygenolytic cleavage only, and (b) other compounds that underwent consecutive reactions after dioxygenation.

Formation of the substituted 2-oxohepta-3,5-dienedioic acids was proven by the mass of the molecular anions (Table V) and by the product ion spectra that showed consecutive losses of CO$_2$ (from one of the carboxylate groups), of CO (from the carbonyl group in 2-position), and sometimes decarboxylation of the second carboxylate group. In the case of the halogenated salicylates, the product ion spectra of the dioxygenation products also showed elimination of HF, HCl, or HBr (Table V).

Some of the initial dioxygenation products were not stable. Dioxygenation of salicylates that are halogenated in the 5-position yielded halogenated carboxylates (4-halo-2-oxo-3,5-hep-
Salicylate 1,2-Dioxygenase from P. salicylatoxidans

TABLE V
Analysis of the dioxygenolytic cleavage of different substituted salicylates by the purified ring-fission dioxygenase by LC-MS/MS

| Reaction a | Parent anion | MS fragments b | UV maximum c |
|------------|-------------|----------------|--------------|
| 5-Cl 6.8   | 1.a         | 167 (–CO₂), 95 (–CO₂–CO) | 302          |
| 8.5        | 1.b         | 185 (–CO₂–CO, 69 (–CO₂–CO₂–CO₃) | 238, 332     |
| 4-Cl 9.5   | 203/205     | 167 (–HCl), 138 (–HCl–CO₂), 128 (–HCl–CO₂–CO), 95 (–HCl–CO₂–CO₂) | 220, 294     |
| 3-Cl 9.1   | 203/205     | 159 (–CO₂), 123 (–CO₂–HCl), 115 (–CO₂–CO₂), 95 (–CO₂–HCl–CO₂) | 304          |
| 4-Br 8.4   | 2           | 159/161 (–HCl), 87 (–CO₂–CO₂–CO) | 372          |
| 10.8       | Unknown     | 201/203 (–HBr), 139 (–HBr–CO₂), 123 (–HBr–CO₂–CO₂ and Br⁻) | 216, 280, 318 |
| 3-Br 9.3   |             | 203 (–CO₂), 159 (–CO₂–CO₂), 123 (–CO₂–HBr), 95 (–CO₂–HBr–CO₂) | 308          |
| 9.0        | 2           | 203/205 (–HBr–CO₂) | 380          |
| 5-F 6.9    | 1.a         | 167 (–CO₂), 95 (–CO₂–CO) | 302          |
| 8.5        | 1.b         | 185 (–CO₂–CO, 69 (–CO₂–CO₂–CO₃) | 236, 332     |
| 5-Me 6.4   |             | 183 (–CO₂–CO), 95 (–CO₂–CO₂) | 208          |
| 8.8        | Unknown     | 205 (–CO₂–CO), 117 (–CO₂–CO₂), 95 (–CO₂–CO₂–CO₂–CO₂) | 216, 300     |
| 5.2        |            | 201 (–H₂O), 157 (–CO₂), 139 (–CO₂–H₂O), 113 (–CO₂–CO₂–CO₂) | Signal too weak |
| 4-Me 5.7   |             | 203 (–H₂O), 159 (–CO₂), 121 (–H₂O–CO₂), 111 (–CO₂–CO₂), 95 (–CO₂–CO₂–CO₂–CO₂) | 214          |
| 3-Me 6.8   |             | 183 (–CO₂–CO), 113 (–CO₂–CO₂), 95 (–CO₂–CO₂–CO₂), 67 (–CO₂–CO₂–CO₂–CO₂) | 215          |
| 5-OH 8.6   |             | 185 (–CO₂–CO₂–CO₂, 69 (–CO₂–CO₂–CO₂) | 238, 332     |
| 4-OH 8.3   |             | 185 (–CO₂–CO₂, 69 (–CO₂–CO₂–CO₂) | 204, 268     |
| 3-OH 8.5   |             | 185 (–CO₂–CO₂) | No spectrum  |
| 4-NH₂ 4.2  |             | 184 (–CO₂), 122 (–CO₂–H₂O), 112 (–CO₂–CO₂), 96 (–CO₂–CO₂–CO₂) | 276          |

a Retention time.
b Numbers refer to the reactions shown in Scheme 2. No remark, initial dioxygenation product.
c In case of halogenated parent ions, m/z values of the lighter isotope.
d Determined by HPLC-DAD in methanolic eluent; if more than one maximum, the highest maximum is shown in boldface.

**Scheme 2.** Consecutive reactions observed for the dioxygenation products of halogenated salicylates. Top, from 5-halo-salicylates; bottom, from 3-halo-salicylates.

tanedienedioic acids) that underwent lactone formation while eliminating HF, HCl, or HBr, as suggested previously (11). Correspondingly, the initial dioxygenation products could not be detected from 5-chloro- and 5-fluorosalicylate by means of LC-MS. Instead, the lactone was the first reaction product that was detected. This lactone was then hydrolyzed to form 4-hydroxy-2-oxo-3,5-heptanedienedioic acid (maleylpyruvate) (Scheme 2, top). The retention time, UV spectrum, and mass spectrometric data of the product generated from both 5-chloro- and 5-fluorosalicylate were identical to those obtained from 5-hydroxyxylate upon dioxygenation. The 4-methyl-2-oxo-3,5-heptadienedioic acid that was generated from 5-methylsalicylate was also unstable and yielded two addition products. One can be ascribed to the addition of water to one of the double bonds (m/z 201), which was consistent with the product ion spectrum in which the loss of water (~18 atomic mass units) was the most pronounced fragmentation. The major product appeared at m/z 205, which was 22 mass units above the initial dioxygenation products. The identity of this product is yet unknown, although its UV spectrum and MS/MS fragmentations are very similar to those observed for the lactone formed from the dioxygenation products of the 5-halo-salicylates (m/z 167; Table V).

The 6-halo-2-oxo-3,5-heptadienedioic acids that were formed by dioxygenation of the 3-halosalicylates were decarboxylated to 6-halo-2-oxo-3,5-hexadienoic acids (Scheme 2, bottom). The electron-withdrawing effect of the halogen in α-position to the 7-carboxylate group may facilitate decarboxylation in these cases. This reaction sequence was detected for the dioxygenation product of 3-chloro- and 3-bromosalicylate (Table V). In case of the hydroxylated (3-, 4-, and 5-), two of the methylated (3- and 4-), and 4-aminosalicylates, the expected products of a 1,2-dioxygenation were observed (Table V).

**Expression Cloning of the Presumed Genitase 1,2-Dioxygenase from C. glutamicum**—As indicated above, the highest degree of sequence identity (58%) was found between the salicylate dioxygenase activity from P. salicylatoxidans and a presumed genitase 1,2-dioxygenase from C. glutamicum. It was therefore tested whether the open reading frame encoded by C. glutamicum indeed encoded a genitase-1,2-dioxygenase and whether this enzyme also converted salicylate. Therefore, the open reading frame was amplified by PCR from the
genomic DNA, and the encoded protein was expressed by using the expression vector pJOE2702 (24, 25). Cell-free extracts from the construct obtained converted gentisate with rather high specific activities (20.6 units mg of protein) but did not convert salicylate or 1-hydroxy-2-naphthoate, although a competitive inhibition of gentisate oxidation in the presence of salicylate was observed ($K_I$ = 2.3 mM).

**DISCUSSION**

The salicylate dioxygenase activity from *P. salicylatoxidans* BN12 is rather unique among the currently known ring fission dioxygenases because the enzyme is able to cleave various substituted salicylates that carry only a single hydroxy group and that are not activated by additional electron-donating substituents for a ring fission reaction. A similar reaction has been described previously only for the ring fission of 1-hydroxy-2-naphthoate, which is an intermediate in the degradation of phenanthrene by certain bacteria. This reaction is catalyzed by a 1-hydroxy-2-naphthoate dioxygenase that results in the formation of trans-2-carboxybenzalpyruvate (10, 12–14). Our previous biochemical characterization of the salicylate dioxygenase activity from *P. salicylatoxidans* BN12 demonstrated that the enzyme converted gentisate, 5-aminosalicylate, and 1-hydroxy-2-naphthoate with much higher catalytic activities compared with salicylate and suggested that the ring fission dioxygenase was also structurally similar to gentisate 1,2-dioxygenases or 1-hydroxy-2-naphthoate dioxygenases. This was indicated by the size of the subunits, the structure of the holoenzyme, and the dependence of the enzyme from Fe$^{2+}$ ions. Nevertheless, it became evident that the ring fission dioxygenase from *P. salicylatoxidans* was clearly different from the presently known gentisate 1,2-dioxygenases or 1-hydroxy-2-naphthoate dioxygenases because of its unique ability to oxidatively cleave salicylate and also the ability to cleave gentisate and 1-hydroxy-2-naphthoate with high catalytic efficiencies (15). In contrast, the 1-hydroxy-2-naphthoate dioxygenase from *Nocardoides* sp. KP7 did not oxidize gentisate or salicylate (12), and gentisate 1,2-dioxygenases do not oxidize salicylate (and presumably also not 1-hydroxy-2-naphthoate) (6).

The previously performed enzymatic analysis did not allow a clear distinction if the ring fission dioxygenase from *P. salicylatoxidans* was more closely related to gentisate 1,2-dioxygenases or to 1-hydroxy-2-naphthoate dioxygenases, although a closer relationship of the enzyme from *P. salicylatoxidans* with gentisate 1,2-dioxygenases was suggested by the observation that the enzyme was composed of four identical subunits that have also been observed for several bacterial gentisate 1,2-dioxygenases (5, 27, 31–35). In contrast, it was suggested that the 1-hydroxy-2-naphthoate dioxygenase from *Nocardoides* sp. KP7 was composed of six subunits (12).

The deduced amino acid sequence of the enzyme obtained in the present study confirmed the results of our previous enzymatic studies because it was found that the enzyme was distantly related to gentisate 1,2-dioxygenases and 1-hydroxy-2-naphthoate dioxygenases from different bacteria. Gentisate 1,2-dioxygenases from *C. glutamicum* ATCC 13032 (GenBank™ accession number NP 602217), *Pseudomonas alcaligenes* NCIMB 9867 (GenBank™ accession number AAD49427), *Sphingomonas* sp. RW5 (GenBank™ accession number CAA12267), and 1-hydroxy-2-naphthoate 1,2-dioxygenase from *Nocardoides* sp. KP7 (GenBank™ accession number BAA31235).
2-naphthoate dioxygenase, and “salicylate-1,2-dioxygenase”) demonstrated the presence of some highly conserved amino acid residues. Thus, it became evident that two histidine pairs (corresponding to His-128/His-130 and His-170/His-172 in the ring fission dioxygenase from \textit{P. salicylatoxidans}) were present in all three enzymes. The conservation of these histidine pairs had been identified previously in a sequence comparison performed with different gentisate 1,2-dioxygenases, and it was shown by site-directed mutagenesis that the replacement of each of these four histidine residues resulted in the inactivation of the gentisate 1,2-dioxygenase from \textit{P. alcaligenes} NCIMB 9867 (36). The conservation of these residues also in the more distantly related enzymes gave further evidence for their importance in catalysis. For the gentisate-1,2-dioxygenase from \textit{P. alcaligenes} NCIMB 9867, it had been suggested that the two pairs of histidines were involved in the binding of the catalytic active ferrous iron (36). It was shown previously for the extradiol dioxygenases, which form the second group of ring fission dioxygenases containing ferrous iron in their catalytic center, that the Fe(II) ions in the catalytic center are bound by a “2-His-1-carboxylate facial triad” and that these three ligands anchor the iron in the active side (37). Thus it may be possible that two of the four conserved histidine residues are involved in the binding of the ferrous iron. In the sequence alignment of the enzyme from \textit{P. salicylatoxidans}, the 1-hydroxy-2-naphthoate dioxygenase, and the gentisate-1,2-dioxygenases (Fig. 4), there also appears one glutamate residue to be highly conserved (corresponding to Glu-200 in the salicylate dioxygenase from \textit{P. salicylatoxidans}), and this residue may be a candidate for the glutamate residue involved in a possible 2-His-1-carboxylate triad.

It was suggested previously that the oxidation of gentisate by gentisate-1,2-dioxygenases (and of homogentisate by the iso-functional homogentisate 1,2-dioxygenases) requires a direct bidentate ligation of the ferrous iron in the catalytic center by the carboxyl and 2-hydroxyl groups of the (homo)gentisate substrate. It was also suggested that the binding of the substrate is followed by a deprotonation of the 5-hydroxyl group of the substrate that presumably involves a histidine residue of the ring fission dioxygenase(s) (6, 38). Although this function may be fulfilled during the oxidation of gentisate by one of the two other highly conserved histidine residues that are not involved in the speculative iron binding described above, this mechanism cannot be involved in the oxidation of (substituted) salicylates catalyzed by the ring fission dioxygenase from \textit{P. salicylatoxidans}, because these substrates are missing a 5-hydroxyl group in their substrates. This suggests that the reaction mechanism for gentisate-1,2-dioxygenases, previously suggested by Harpel and Lipscomb (6), which required the isomerization of a 5-hydroxy (or 5-amino) group to the keto (or imino) resonance form during the catalytic cycle (Scheme 3), will probably need some revision. The observation that in \textit{C. glutamicum} a gentisate dioxygenase is present, which shows a high degree of sequence identity (58%) with the salicylate dioxygenase activity but which is not able to convert salicylate, may allow the identification of those specific amino acid substitutions that allow the enzyme from \textit{P. salicylatoxidans} to attack salicylate.

The tests with a wide range of substituted salicylates confirmed our previous observation that gentisate and 5-aminosalicylate were converted with extraordinary high \(V_{\text{max}}\) values compared with the other substrates, because all other salicylates available were oxidized only with less than 10% of the value found with gentisate. On the other hand, it became evident that 3- and 5-aminosalicylates were only bound with a very low affinity to the enzyme and that the \(K_m\) values with these substrates were at least 10 times higher than those found with the other substrates. A much higher \(K_m\) value for 5-aminosalicylate compared with gentisate as substrate has also been observed previously for the gentisate 1,2-dioxygenases from “\textit{Pseudomonas} testosteroni” and “\textit{Pseudomonas} acidovorans” as described by Harpel and Lipscomb (6). This may suggest some kind of “repulsion” of an amino group in the 5-position of the respective substrate from the relevant part of the enzyme involved in the binding of the hydroxyl group of the natural substrate gentisate.

A comparison of the \(V_{\text{max}}\) values observed with the halogenated salicylates carrying the respective substituents in the 5-position demonstrated a clear preference for 5-fluorosalicylate compared with 5-chloro- and 5-bromosalicylate. Furthermore, it was found that the \(V_{\text{max}}\) value for 5-fluorosalicylate was higher than that observed with salicylate. This suggested that at least for salicylates carrying a substituent in the 5-position, the electronic effect of the highly electronegative fluorine atom did not significantly decrease the reaction rates and that the increasing size of the halogen atoms in the series fluorne, chlorine, and bromine were mainly responsible for the decreasing reaction rates if the substituent was changed from fluorine, via chlorine, to the bromine.

Many of the highly oxidized and highly unsaturated products generated from substituted salicylates by the 1,2-dioxygenase were chemically unstable and underwent elimination and addition reactions. These reactions were governed by the type and the position of the substituents. Thus, the LC/MS analysis clearly demonstrated that the products formed by the 1,2-dioxygenation of 3-halo-salicylates underwent decarboxylation. On the contrary, the conversion of 5-halo-salicylates resulted in a dehalogenation via lactone formation. A similar dehalogenation process has been suggested previously for the degradation of 5-chlorosalicylic acid by a \textit{“Bacillus”} that was isolated from the Mississippi River (11). Thus, the dioxygenation of 5-substituted halogenated salicylates ultimately leads to a dehalogenation reaction and may convert xenobiotic substrates into intermediates also formed during the degradation of natural compounds.
REFERENCES

1. Que, L., Jr. & Ho, R. Y. N. (1996) Chem. Rev. 96, 2607–2624
2. Bugg, T. D. H. & Lin, G. (2001) Chem. Commun. 941–952
3. Mishra, V., Lal, R. & Srinivisan, (2001) Crit. Rev. Microbiol. 27, 133–166
4. Davis, J. K., He, Z., Sumerville, C. C. & Spain, J. C. (1999) Arch. Microbiol. 172, 330–339
5. Harpel, M. R. & Lipscomb, J. D. (1990) J. Biol. Chem. 265, 6301–6311
6. Harpel, M. R. & Lipscomb, J. D. (1990) J. Biol. Chem. 265, 22187–22196
7. Lendenmann, U. & Spain, J. C. (1996) J. Bacteriol. 178, 6227–6232
8. Stolz, A., No¨rtemann, B. & Knackmuss, H.-J. (1992) Biochem. J. 282, 675–680
9. Takenaka, S., Murakami, S., Shinke, R., Hatakeyama, K., Yukawa, H. & Aoki, K. (1997) J. Biol. Chem. 272, 14727–14732
10. Iwabuchi, T. & Harayama, S. (1998) J. Bacteriol. 178, 8332–8336
11. Crawford, R. L., Olson, P. E. & Frick, T. D. (1979) Appl. Environ. Microbiol. 38, 379–384
12. Iwabuchi, T., & Harayama, S. (1998) J. Biol. Chem. 273, 8332–8336
13. Kiyohara, H. & Nakao, K. (1977) Agric. Biol. Chem. 41, 705–707
14. Kiyohara, H. & Nakao, K. (1978) J. Gen. Microbiol. 105, 69–75
15. Hintner, J.-P., Lechner, C., Riegert, U., Kuhm, A. E., Storm, T., Reemtsma, T. & Stolz, A. (2001) J. Bacteriol. 183, 6936–6942
16. Kumpfer, P., Muller, C., Mau, K., Neef, A., Auling, G., Busse, H.-J., Osborn, A. M. & Stolz, A. (1999) Int. J. Syst. Bacteriol. 49, 887–897
17. Nortemann, B. (1987) Bakterieller Abbau von Amino- und Hydrazynaphtha-
linsauren, Ph.D. Thesis, University of Stuttgart, Germany
18. Dorn, E., Hellwig, M., Reineke, W. & Knackmuss, H.-J. (1974) Arch. Microbiol. 98, 61–70
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
20. Stone, K. L., LiPresti, M. B., Crawford, J. M., DeAngelis, R. & Williams, K. R. (1989) in A Practical Guide to Protein and Peptide Purification for Micro sequencing (Matsudaira, P. T., ed) pp. 31–47, Academic Press, Inc. San Diego
21. Laemmli, U. K. (1970) Nature 227, 680–685
22. Pang, K. M. & Knecht, D. A. (1997) BioTechniques 22, 1046–1048
23. Marchuk, D., Drumm, M., Saulino, A. & Collins, F. S. (1991) Nucleic Acids Res. 19, 1154
24. Volf, J. N., Eichenseer, C., Viell, P., Piendl, W. & Altenbuchner, J. (1996) Mol. Microbiol. 21, 1057–1047
25. Stumph, T., Wilms, B. & Altenbuchner, J. (2000) BioSpekttrum 6, 33–36
26. Rubio, M. A., Engesser, K.-H. & Knackmuss, H.-J. (1986) Arch. Microbiol. 145, 116–122
27. Feng, V., Kho, H. E. & Poh, C. L. (1999) Appl. Environ. Microbiol. 65, 946–950
28. Scott, A. I. (1964) Interpretation of the Ultraviolet Spectra of Natural Products, pp. 45–88, Pergamon Press Ltd., Oxford
29. Takenaka, S., Murakami, S., Shinke, R. & Aoki, K. (1998) Arch. Microbiol. 170, 132–137
30. Kucharczyk, R., Zagulsi, M., Rytka, J. & Herbert, C. J. (1998) FEBS Lett. 424, 127–130
31. Crawford, R. L., Hutton, S. W. & Chapman, P. J. (1975) J. Bacteriol. 121, 794–799
32. Fu, W. & Oriel, P. (1998) Extremophiles 2, 439–446
33. Suarez, M., Ferrer, E. & Martin, M. (1996) FEMS Microbiol. Lett. 143, 89–95
34. Werwath, J., Arfmann, H.-A., Pieper, D. H., Timmis, K. N. & Wittich, R.-M. (1998) J. Bacteriol. 180, 4171–4176
35. Zhou, N.-Y., Fuenmayor, S. L. & Williams, P. A. (2001) J. Bacteriol. 183, 700–708
36. Chua, C. H., Feng, Y. Y., Khoo, H. E. & Poh, C. L. (2001) FEMS Microbiol. Lett. 204, 141–146
37. Hegg, E. C. & Que, L., Jr. (1997) Eur. J. Biochem. 250, 625–629
38. Titus, G. P., Mueller, H. A., Burgner, J., de Cordoba, S. R., Pehalva, M. A. & Timm, D. E. (2000) Nat. Struct. Biol. 7, 542–546