Arch Microbiol (2007) 187:475–487
DOI 10.1007/s00203-007-0215-z

ORIGINAL PAPER

A new monocupin quercetinase of *Streptomyces* sp. FLA: identification and heterologous expression of the *queD* gene and activity of the recombinant enzyme towards different flavonols

Hedda Merkens · Sonja Sielker · Karsten Rose · Susanne Fetzner

Received: 11 March 2006 / Revised: 18 November 2006 / Accepted: 11 January 2007 / Published online: 14 February 2007

Abstract The gene *queD* encoding quercetinase of *Streptomyces* sp. FLA, a soil isolate related to *S. eurythermus*^{T}, was identified. Quercetinases catalyze the 2,4-dioxygenolytic cleavage of 3,5,7,3′,4′-pentahydroxyflavone to 2-protocatechuoylphloroglucinol carboxylic acid and carbon monoxide. The *queD* gene was expressed in *S. lividans* and *E. coli*, and the recombinant hexahistidine-tagged protein (QueDHis$_6$) was purified. Several flavonols were converted by QueDHis$_6$, whereas CO formation from the 2,3-dihydroflavonol taxifolin and the flavone luteolin were not observed. In contrast to bicupin quercetinases from *Aspergillus japonicus* and *Bacillus subtilis*, and bicupin pirins showing quercetinase activity, QueD of strain FLA is a monocupin exhibiting 35.9% sequence identity to the C-terminal domain of *B. subtilis* quercetinase. Its native molecular mass of 63 kDa suggests a multimeric protein. A *queD*-specific probe hybridized with fragments of genomic DNA of four other quercetin degrading *Streptomyces* strains, but not with DNA of *B. subtilis*. Potential ORFs upstream of *queD* probNSably code for a serine protease and an endoribonuclease; two ORFs downstream of *queD* may encode an amidohydrolase and a carboxylesterase. This arrangement suggests that *queD* is not part of a catabolic gene cluster. Quercetinases might play a major role as detoxifying rather than catabolic enzymes.

Keywords Flavonoid · Flavonol · Quercetin · Quercetinase · Dioxygenase · Monocupin · Pirin · Polyphenol · Degradation · *Streptomyces*

Abbreviations

aa	Amino acids
acc no	Accession number
ATCC	American type culture collection
DMSO	Dimethyl sulfoxide
DSM	Deutsche Sammlung von Mikroorganismen
IPTG	Isopropyl-β-D-thiogalactopyranoside

Introduction

Flavonoids are polyphenolic substances which are synthesised by a wide range of vascular plants (Iwashina 2000), acting as visual attractors, photoreceptors, feeding repellents, antimicrobials, and antioxidants (Pietta 2000). The flavonol quercetin (3,5,7,3′,4′-pentahydroxyflavone) is found in considerable amounts in vegetables and fruits like onions, broccoli, and apples (Duthie et al. 2003) and is an important constituent of the human diet. A number of health benefits are attributed to this plant polyphenol mainly due to its antioxidant properties (Pietta 2000; Prior 2003), and it is considered to be a potential anti-cancer agent (Lamson and Brignall 2000).

Microbial degradation of quercetin has been reported for fungi, e.g., *Aspergillus* spp., *Pullularia* sp.,

H. Merkens · S. Sielker · K. Rose · S. Fetzner (✉)
Institut für Molekulare Mikrobiologie und Biotechnologie,
Westfälische Wilhelms-Universität Münster,
Corrensstr. 3, 48149 Münster, Germany
e-mail: fetzner@uni-muenster.de

Present Address:
K. Rose
Institut für Pharmazeutische und Medizinische Chemie,
Westfälische Wilhelms-Universität Münster,
Hittorfstr. 58-62, 48149 Münster, Germany

and *Fusarium oxysporum* (Westlake et al. 1961; Barz 1971) and for bacteria such as *Pseudomonas putida*, *Rhizobium* spp., and *Actinoplanes missouriensis* (Schultz et al. 1974; Rao and Cooper 1994; Rose and Fetzner 2006). Intestinal *Clostridium* species and *Eubacterium ramulus* (Winter et al. 1989; Schneider and Blaut 2000) as well as methanogenic consortia (Herrmann et al. 2001) anaerobically convert the flavonoid.

In the initial step of aerobic degradation, quercetinase catalyzes the 2,4-dioxygenolytic cleavage of quercetin to form 2-protocatechuoylphoroglucinol carboxylic acid and carbon monoxide. Quercetinases have so far been isolated from *Aspergillus flavus* (Oka et al. 1972), *A. niger* (Hund et al. 1999), *A. japonicus* (Fusetti et al. 2002), and *Bacillus subtilis* (Bowater et al. 2004; Barney et al. 2004), and the corresponding genes of *A. japonicus* and *B. subtilis* have been characterized (Kooter et al. 2002; Bowater et al. 2004). The crystal structures of *A. japonicus* and *B. subtilis* quercetinase revealed that both enzymes belong to the cupin superfamily (Fusetti et al. 2002; Gopal et al. 2005).

The cupin fold consists of a characteristic β-barrel domain (lat. *cupa*: small barrel), which comprises two conserved amino acid motifs $G(X)_5HXH(X)_{3,4}E(X)_6G$ and $G(X)_5PXG(X)_2H(X)_3N$, separated by an inter-motif region that varies in length from 11 to >100 aa (amino acid) residues. Depending on the number of cupin domains present, the superfamily is subdivided into monocupins (single cupins), bicupins, and multicupins (Dunwell et al. 2000, 2001, 2004). The active site of enzymatic members is located in the centre of the β-barrel and includes four highly conserved residues (two His and Glu in motif 1, His in motif 2), which can bind different metal ions. As the homodimeric quercetinases of *A. japonicus* and *B. subtilis* exhibit two cupin domains per monomer, they are both counted among the bicupin subset. Whereas the fungal enzyme contains Cu^{2+} (Oka et al. 1972; Kooter et al. 2002), the *Bacillus* enzyme is presumed to prefer Mn^{2+} as cofactor (Schaab et al. 2006), despite having been purified as an iron enzyme from a recombinant *E. coli* clone (Bowater et al. 2004; Barney et al. 2004).

Streptomycetes are wide-spread soil bacteria which play an important role in the decomposition of biopolymers such as lignin, cellulose, hemicellulose, chitin, keratin, and pectin (Locci 1989). Lignin monomers and related compounds like *trans*-cinnamic acid, *p*-coumaric acid, ferulic acid, or vanillin are also utilized by members of this genus (Sutherland et al. 1983). Modification reactions of flavonoids, such as regiospecific hydroxylation and O-methylation, were described for several *Streptomyces* spp. (Hosny et al. 2001; Yoon et al. 2005; Kim et al. 2006), but knowledge on the potential of Streptomycetes to degrade flavonoids is very limited. In 1959, Westlake et al. (1959) reported that 24 out of 51 *Streptomyces* spp. tested were able to utilize rutin (quercetin 3-*O*-glycoside); however, the reactions and enzymes involved have not been investigated as yet.

Here, we report the detection of a CO-forming quercetinase in *Streptomyces* sp. strain FLA, the identification and heterologous expression of the corresponding gene designated *queD* (for quercetin dioxygenase), and the purification and characterization of recombinant QueD. In contrast to quercetinases from *Bacillus subtilis* and *Aspergillus japonicus*, QueD from this *Streptomyces* strain belongs to the monocupin family, which raises interesting questions about the evolution of bacterial and fungal quercetinases.

Materials and methods

Bacterial strains and plasmids, culture conditions, and preparation of *Streptomyces* protoplasts

Streptomyces sp. designated strain FLA (for flavonol utilization) was isolated from a soil sample collected near Stuttgart, Germany, by enrichment on quercetin agar. *S. alboniger* DSM 40043, *S. aureofaciens* ATCC 10762, *S. avermitilis* MA-4680 (DSM 46492), *S. cinereoruber* DSM 40012, *S. cinnamoneus*, *S. coelicolor* A3(2) (DSM 40783; *S. violaceoruber*), *S. echinatus* Tü 12, *S. eurythermus* DSM 40014, *S. flaveolus* Tü 55, *S. fradiae* T59-235, *S. glaucus* Tü 490, *S. griseoflavus* Tü 52, *S. lividans* TK23, *S. prasinopilosus* DSM 40098, *S. prasinus* DSM 40099, *S. tendae* Tü 901, *S. toxytricini* DSM 40178, and *S. viridochromogenes* DSM 40110 were also tested for quercetin consumption. Strains and plasmids used for cloning and expression of *queD* are listed in Table 1. *Streptomyces* and *Escherichia coli* strains were grown at 30°C in Standard I medium (Merck, Darmstadt, Germany) and at 37°C in Lysogeny Broth (LB; Sambrook et al. 1989), respectively; LB contained ampicillin (100 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}), if appropriate.

Streptomyces strains were inoculated onto overlay agar plates with mineral salts medium (Schlegel et al. 1961) and 2 mM quercetin in the upper layer to investigate quercetin consumption. For the preparation of cell extracts to determine quercetinase activity, *Streptomyces* strains were grown in Standard I medium for 2 days before 1 mM quercetin was added. After another 4 h of incubation, cells were harvested by centrifugation.

Table 1 Bacterial strains and plasmids used for cloning of *queD* from *Streptomyces* sp. FLA

Strain/plasmid	Characteristics	Reference
S. lividans TK 23	*spc-1* SLP2$^-$ SLP3$^-$	Hopwood et al. (1985)
Escherichia coli DH5α	F′/*endA1 hsdR17* (r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA* (Nal^r) *relA1 Δ*(*lacIZYA-argF*)*U169 deoR* (ϕ80*dlacΔ*(*lacZ*)*M15*)	Grant et al. (1990)
Escherichia coli BL21 (DE3) pLysS	F$^-$, *ompT*, *hsdS*$_β$($r_β^-$ $m_β^-$), *dcm*, *gal*, (DE3), pLysS(Cm^R)	Novagen
pIJ702	pIJ101 derivative, *tsr mel*	Katz et al. (1983)
pIJ702HM	3.4-kb *Bam*HI fragment of genomic DNA of *Streptomyces* sp. FLA in *Bgl*II site of pIJ702	This study
pUC18	ColE1 *lacZ*, *bla*	Vieira and Messing (1982)
pUC18KQ	2.9-kb *Kpn*I fragment of DNA of strain FLA in *Kpn*I site of pUC18	This study
pUC18BQ	3.4-kb *Bam*HI fragment of DNA of strain FLA in *Bam*HI site of pUC18	This study
pET23a	Expression vector, T7 promoter, *bla*	Novagen
pET23a*queD*	*queD* gene of strain FLA in *Nde*I and *Hind*III site of pET23a	This study

To assess growth of *Streptomyces* sp. FLA on quercetin as a sole source of carbon and energy, equal amounts of a washed cell suspension were used to inoculate mineral salts medium (Schlegel et al. 1961) containing 1 mM quercetin. Cultures were grown for approximately 5 weeks until decolorisation of the yellow medium indicated consumption of quercetin, and dry weight of biomass was determined. Mineral salts medium containing 1% glucose and medium lacking any carbon source, inoculated with the same amount of cells, were used as controls.

For the preparation of quercetinase, *Streptomyces* sp. FLA was grown in GYM medium (0.4% glucose, 0.4% yeast extract, 1% malt extract, pH 7.2) for 2 days at 30°C, then 1 mM quercetin was added and the culture was incubated for another 2 days at 25°C. Cells were harvested, washed twice with saline (0.5% NaCl, 0.012% $MgSO_4 \cdot 7H_2O$), resuspended in mineral salts medium containing 2 mM quercetin, and harvested after two more days of growth at 25°C.

To examine whether the presence of glucose as an additional carbon source besides quercetin affects synthesis of quercetinase, *Streptomyces* sp. FLA was grown in Standard I medium for 24 h, 1 mM quercetin was added, and cells were harvested by centrifugation after another 24 h of incubation. Cell pellets were washed twice in saline, resuspended in mineral salts medium (Schlegel et al. 1961) containing 2 mM quercetin or 2 mM quercetin and 1% glucose, incubated for 24 h at 30°C, and harvested.

For preparation of protoplasts, *S. lividans* TK23 was cultivated in YEME-medium; R5 agar plates were used for protoplast regeneration after transformation. These media and the method used for protoplast preparation are described in Kieser et al. (2000). Media for growth of *S. lividans* TK23 harbouring pIJ702 or derivatives contained thiostrepton (25 μg ml^{-1}). To identify clones with quercetinase activity, *S. lividans* TK23 was inoculated onto overlay agar plates with mineral salts medium (Schlegel et al. 1961) and 2 mM quercetin in the upper layer.

Preparation of crude extracts, estimation of protein concentration, and enzyme assays

Streptomyces cells were resuspended in 50 mM Tris/HCl buffer, pH 7 (pH 9 for protein purification from wild-type strain FLA) and disrupted by sonication. Cell debris was removed by centrifugation. Protein concentrations were determined according to the method of Zor and Selinger (1996), using bovine serum albumin as standard.

Quercetinase activity was determined spectrophotometrically by measuring quercetin consumption. The reaction mixture in a total volume of 1 ml contained 50 μl of 1.2 mM quercetin dissolved in dimethyl sulfoxide (DMSO) and appropriate amounts of protein in 50 mM Tris/HCl buffer, pH 7.0 (for wild-type quercetinase) or pH 8.0 (for recombinant quercetinase). One unit was defined as the amount of enzyme that converts 1 μmol of quercetin per min at 25°C in these buffers ($\varepsilon_{367\,nm,\ pH8} = 14{,}850\ M^{-1}\ cm^{-1}$; $\varepsilon_{367\,nm,\ pH7} = 17{,}100\ M^{-1}\ cm^{-1}$). All kinetic assays were carried out in air-saturated buffer. For the determination of kinetic constants for quercetin, substrate concentrations of 1.75–150 μM were used, and apparent K_m and k_{cat} values were deduced from Hanes plots (Hanes 1932). Assays were done at least in triplicate.

Enzyme-catalyzed formation of carbon monoxide from quercetin was detected as described by Waterman (1978). Protein solution (300 µl), 1.2 mM quercetin dissolved in DMSO (200 µl), and 400 µl of 50 mM Tris/HCl buffer, pH 7.0 were mixed and incubated for 3 min. After subsequent addition of 50 µl hemoglobin (30 mg ml^{-1} in H_2O) and 50 µl sodium dithionite (50 mg ml^{-1} in H_2O), a visible absorption spectrum was recorded (450–650 nm). CO-hemoglobin exhibits two absorption maxima at 540 and 570 nm, whereas reduced O_2-hemoglobin shows a single maximum at 552 nm.

The activity of purified recombinant quercetinase towards flavonoids was determined by measuring oxygen consumption with a Clark-type oxygen electrode (Digital Model 10, Rank Brothers Ltd, Cambridge, England). The assay contained 50 µl of a flavonoid solution (1 mM in DMSO), 20 µl enzyme (0.0112–1.12 U), and 930 µl 50 mM Tris/HCl buffer, pH 7; such pH was chosen because myricetin rapidly decomposed at pH 8. To detect quercetinase-catalyzed CO production from flavonoids, the same assay was carried out in a 1.5 ml reaction tube, with a filter paper soaked with aqueous $PdCl_2$ (1:500, w/v) placed in the lid. CO released by the enzyme-catalyzed reaction reduces Pd^{2+} to elemental palladium, which precipitates as a black solid (Arendt and Dörmer 1972).

Preparation of quercetinase from strain FLA and determination of its N-terminal amino acid sequence

Ammonium sulfate was added to crude extract supernatant of strain FLA at 4°C to 10% saturation, and the supernatant after centrifugation for 20 min at 48,000*g* was loaded onto a phenyl-Sepharose CL-4B column (20 ml) (Amersham Biosciences, Freiburg, Germany) that had been equilibrated in 50 mM Tris/HCl buffer containing 0.4 M $(NH_4)_2SO_4$, pH 9.0. After two washing steps with 0.4 and 0.1 M $(NH_4)_2SO_4$, proteins were eluted with a linear gradient (20 ml) from 0.1 to 0 M $(NH_4)_2SO_4$ in equilibration buffer. Fractions showing quercetinase activity were pooled and chromatographed at a 1 ml Uno™-Q column (BioRad, München, Germany) that had been equilibrated in 50 mM Tris/HCl buffer, pH 9.0. Proteins were eluted with a linear gradient (25 ml) from 0 to 0.28 M NaCl in the equilibration buffer. The active fractions were combined and concentrated by ultra-filtration. The concentrate was separated in a preparative native 7.5% polyacrylamide gel ("high pH discontinuous system" according to Hames 1990). The active protein was eluted from the gel by dialysis, concentrated by ultra-filtration, and stored at −80°C.

For N-terminal sequencing, the quercetinase preparation was separated in a 12.5% SDS-polyacrylamide gel (Laemmli 1970) and transferred to a polyvinylidene fluoride membrane (Millipore, Eschborn, Germany) by semi-dry electroblotting (Towbin et al. 1979). After staining (0.1% Coomassie Brilliant Blue R-250 in 50% methanol, 7% acetic acid), destaining (50% methanol, 7% acetic acid) and drying of the membrane, the amino-terminal sequence of the 21 kDa protein was determined by automated Edman degradation by Dr. Bernhard Schmidt (Zentrum Biochemie und Molekulare Zellbiologie, Biochemie II, Georg-August-Universität Göttingen, Germany).

DNA techniques

Chromosomal DNA of *Streptomyces* sp. FLA was prepared as described by Pospiech and Neumann (1995). Plasmid DNA was isolated with the E.Z.N.A.® Plasmid Miniprep Kit (PeqLab, Erlangen, Germany). Gel extraction of DNA fragments for cloning was done with the E.Z.N.A.® Gel Extraction Kit (PeqLab, Erlangen, Germany). DNA restriction, dephosphorylation, ligation, and agarose gel electrophoresis were carried out using standard procedures (Sambrook et al. 1989). Preparation of competent *E. coli* DH5α and BL21 (DE3) pLysS cells and transformation was performed as described by Hanahan (1983).

Polymerase chain reaction (PCR) for amplification of *Streptomyces* DNA was performed using *Pfu* DNA polymerase (Fermentas, St. Leon-Roth, Germany), with glycerol added to the reaction mixture to a final concentration of 5%. For strain identification, 16S rDNA of *Streptomyces* sp. FLA was amplified by PCR using the primers GM3F and GM4R (Muyzer et al. 1995). DNA sequencing was performed by MWG-Biotech AG (Ebersberg, Germany).

DNA probes and hybridization

Southern and colony blotting, hybridization, and colorimetric detection with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate were carried out following the DIG System User´s Guide for Filter Hybridization (Roche molecular biochemicals, 1995). The degenerated oligonucleotide probe Q1 (5′-AC(G/C)ATCGA(A/G)TACGC(G/C)AC(G/C)CG(G/C)CACCG(G/C)GC(G/C)CG-3′) was synthesized based on the N-terminal amino acid sequence of quercetinase (TIEYATRHRAR) and 3′-labeled with digoxigenin (MWG-Biotech AG, Ebersberg, Germany). To generate the specific probe Q2, a 377 bp fragment of the quercetinase gene was amplified by PCR using the

primers 5′-GACCATCGAATACGCCACC-3′ and 5′-CGACCTGCGAGTGGTGGC-3′, and pUC18KQ (Table 1) as template. The PCR product was purified with the High Pure PCR Product Purification Kit and digoxigenin-labeled with the DIG-High Prime Kit (both kits from Roche, Mannheim, Germany). Prehybridization for 2 h and hybridization overnight with Q1 or Q2 were carried out at 68°C.

Construction of genomic libraries

To generate enriched gene libraries for *Streptomyces* sp. FLA, *Kpn*I- or *Bam*HI-restricted genomic DNA was separated in 1.0% agarose gels and vacuum-blotted to nylon membranes (Porablot NY plus from Macherey-Nagel, Düren, Germany). Fragments in the size of 2.5–3.5 kb (*Kpn*I) and 3.0–4.0 kb (*Bam*HI), showing positive hybridization signals with probes Q1 and Q2, respectively, were extracted from preparative agarose gels and ligated into linearised and dephosphorylated vector pUC18. *E. coli* DH5α transformants were screened by colony blotting using probes Q1 (*Kpn*I library) and Q2 (*Bam*HI library).

Sequence analysis

Gene-coding sequences were identified using the program FramePlot version 2.3.2 (Ishikawa and Hotta 1999), which considers the high G + C distribution at the third position of *Streptomyces* genes. Database searches were carried out at the NCBI with the BLAST family of programs (Altschul et al. 1990) from the NCBI website (http://www.ncbi.nlm.nih.gov). Conserved protein domain sequences were found by CD-Search (Marchler-Bauer et al. 2005). For calculating similarities and identities, and for compiling multiple alignments, the programs GAP (Needleman and Wunsch 1970) and ClustalW (Higgins et al. 1996) were used, respectively. Secondary structure and signal peptide predictions were carried out using PredictProtein (Rost et al. 2004) and both SignalP and TatP (Bendtsen et al. 2004, 2005), respectively.

Expression of *queD* in *Streptomyces lividans* TK23

For heterologous expression of *queD* from its own promoter, the 3.4 kb *Bam*HI insert of pUC18BQ was ligated into the *Bgl*II digested *Streptomyces* clonig vector pIJ702, resulting in pIJ702HM. Polyethylene glycol-assisted protoplast transformation, carried out using the "rapid small-scale procedure" described in Kieser et al. (2000), was used to transfer the recombinant plasmid to *S. lividans* TK23. The transformants were screened on quercetin overlay agar plates. Quercetinase activity was indicated by formation of zones of decolorisation on the yellow agar plates.

Cloning and overexpression of *queD* in *E. coli*

The gene *queD* was amplified by polymerase chain reaction using pUC18BQ as a template and the primers 5′-GGAATTCCATATGACCATCGAATACGCCAC-3′ and 5′-CCC*AAGCTT*CCTTCCCTCGATACTCCCGGTGTGCCACTG-3′, which insert a *Nde*I site (underlined) at the start codon, and a *Hind*III site (underlined and in italics) at the stop codon. After digestion with *Nde*I and *Hind*III, the PCR product was ligated into the vector pET23a, restricted with the same enzymes, resulting in pET23a*queD*. Sequencing of both strands of the insert of pET23a*queD* confirmed that its sequence was identical to that of the template. *E. coli* BL21 (DE3) pLysS was transformed with pET23a*queD* and the recombinant strain was grown to an optical density at 600 nm of 0.5, before expression of *queD* was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The temperature was reduced to 25°C, and after 6 h of induction, cells were harvested by centrifugation at 10,000*g* for 10 min at 4°C and stored at −80°C. Gene expression from the T7 promoter of pET23a produces QueD protein with a C-terminal hexahistidine-tag, designated QueDHis$_6$.

Purification of recombinant QueDHis$_6$

E. coli BL21 (DE3) pLysS pET23a*queD* cells were resuspended in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM $MgCl_2$, and lysed by the pLysS-encoded lysozyme. After DNA digestion for 1.5 h at 4°C using 25 units Benzonase (Novagen) per ml, the extract was centrifuged at 39,000*g* for 30 min at 4°C, and imidazole and NaCl were added to the supernatant to a final concentration of 10 and 300 mM, respectively. This protein solution was loaded onto a MT5 column (BioRad), packed with Ni-NTA Agarose (Qiagen, Hilden, Germany) that had been equilibrated in 50 mM Tris/HCl buffer containing 20 mM imidazole and 300 mM NaCl (pH 7.5). Recombinant QueDHis$_6$ was eluted from the column with a linear gradient (20 ml) of imidazole (20–500 mM) in the same buffer. Fractions exhibiting quercetinase activity were pooled, washed with 50 mM Tris/HCl buffer, pH 8, and concentrated by ultrafiltration. After addition of 10% (v/v) glycerol, the concentrate was stored at −80°C. To determine the purity of the enzyme, it was subjected to SDS-PAGE (12.5% acrylamide gel according to Laemmli (1970)). Polyacrylamide gels were stained with Coomassie Brilliant Blue

G-350 (0.15% Coomassie Brilliant Blue G-350 (w/w), 0.25% $CuSO_4 \cdot 5H_2O$ (w/w), 45% methanol (v/v), 10% (v/v) acetic acid), and destained in an aqueous solution of 30% (v/v) methanol and 10% (v/v) acetic acid.

Gel filtration of $QueDHis_6$ for estimation of its native molecular mass was performed on a Bio-Prep SE-1000/17 column (BioRad Laboratories) in 50 mM Tris/HCl buffer, pH 7.5 containing 150 mM NaCl. Gel Filtration Standard marker from BioRad was used for calibration of the column.

Nucleotide sequence accession numbers

The sequences of the 4.5 kb *Kpn*I/*Bam*HI fragment that includes *queD* and of the 16S rDNA of *Streptomyces* sp. FLA have been deposited in the EMBL Nucleotide Sequence Database under accession numbers AM234612 and AM234613, respectively.

Results

Quercetin-degrading Streptomycetes, and characterization of *Streptomyces* sp. strain FLA

Out of 18 *Streptomyces* spp. tested besides strain FLA, the strains *S. alboniger* DSM 40043, *S. eurythermus* DSM 40014, *S. flaveolus* Tü 55, and *S. tendae* Tü 901 were able to decompose the flavonol quercetin as indicated by formation of colorless zones on quercetin agar. Comparison of the 16S rDNA gene sequence of strain FLA revealed significant homology to *S. eurythermus*^{T} DSM 40014 (99.46% identity within 1,482 nucleotides), *S. nogalater*^{T} DSM 40546 (99.18% identity within 1,469 nucleotides), and *S. tendae*^{T} DSM 40101 (98.38% identity within 1,483 nucleotides). *Streptomyces* sp. FLA is able to utilize quercetin as sole carbon source; however, growth is very poor. After approximately 5 weeks of growth, the measured gain of cell dry weight, compared with cells incubated without any carbon source, was only 1.8 ± 0.3 mg. Such poor increase in biomass was not due to limitation by other components of the mineral salts medium, since strain FLA showed abundant growth in the same medium containing 1% glucose.

Quercetinase of *Streptomyces* sp. FLA

After induction of *Streptomyces* sp. strain FLA with quercetin, specific quercetinase activities between 0.17 and 0.19 U mg^{-1} were measured in crude extracts (soluble fraction). The four-step purification procedure resulted in a 590-fold enrichment of quercetinase from crude extract supernatant in 27% yield. About 0.2 mg protein with a specific activity of 98 U mg^{-1} were obtained from 38 g of wet biomass of strain FLA. Enzyme-catalyzed quercetinase conversion resulted in release of carbon monoxide, as shown by detection of CO-hemoglobin. When quercetin was omitted from the growth medium, quercetinase activity was not detected in crude extracts, indicating that synthesis of the enzyme is induced by the flavonol. The presence of glucose as a second carbon source in the medium besides quercetin did not significantly influence quercetinase activity in cell extract supernatants, suggesting that there is no carbon catabolite control of quercetin metabolism by glucose.

Cloning of the quercetinase gene *queD* of *Streptomyces* sp. FLA

A 2.9 kb *Kpn*I fragment of total DNA of *Streptomyces* sp. FLA specifically hybridized with probe Q1. DNA fragments sized between 2.5 and 3.5 kb were ligated into *Kpn*I-digested pUC18, and colony hybridization of the corresponding library in *E. coli* DH5α using the same probe resulted in the identification of a clone harbouring pUC18KQ (Table 1). Since sequence analysis of the 2.9 kb insert revealed that it only contained part of the presumed quercetinase gene, the obtained sequence was used to synthesize specific probe Q2, which was used to screen a *Bam*HI library, yielding an *E. coli* clone that contained the complete quercetinase gene within a 3.4 kb fragment (pUC18BQ). It is interesting to note that the Q2 probe also specifically hybridized to distinct fragments of genomic DNA of the *S. alboniger*, *S. eurythermus*, *S. flaveolus*, and *S. tendae* strains that convert quercetin, whereas no hybridization signals were observed with DNA of *Bacillus subtilis*, even when attenuating the stringency of hybridization (data not shown).

Sequence analysis of *queD* and flanking regions

From the *E. coli* clones harbouring pUC18KQ and pUC18BQ (Table 1), a total DNA sequence of 4.5 kb was obtained, which was predicted to comprise three full and two truncated ORFs (Fig. 1). The hypothetical protein fragment encoded by truncated ORF1 is related to corresponding C-terminal segments of a broad range of proteases, e.g., to tripeptidylaminopeptidase Tap (acc no AAA92338) from *Streptomyces lividans* 66 (36% identity). The similarity includes a conserved GxSxG "nucleophile elbow" motif whose serine is potentially involved in the catalytic action of

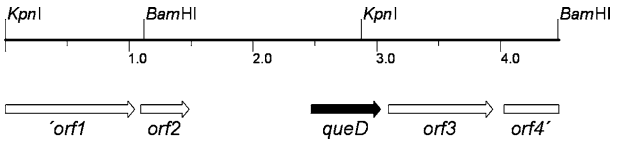


Fig. 1 DNA region (4.5 kb) of *Streptomyces* sp. FLA containing the gene coding for quercetinase (*queD*). Putative ORFs are indicated by *open arrows*. For a description, see text

this protease (Krieger et al. 1994; Butler et al. 1995) and of related α/β-hydrolases, suggesting that ORF1 encodes a serine hydrolase.

Putative ORF2 is located 1,378 nucleotides upstream of *queD* and is deduced to code for a protein of 130 aa with a calculated molecular mass of 14,373 Da. The ORF2 protein shows close similarity to endoribonuclease L-PSP of *Nitrosospira multiformis* ATCC 25196 (acc no ABB75555, 129 aa, 69% identity). The amino acid segment spanning positions 18–126 is related to the conserved domain of the L-PSP (rat liver perchloric acid-soluble protein) endoribonucleases (Pfam signature PF01042). Members of this family are thought to inhibit protein synthesis by cleavage of mRNA (Morishita et al. 1999) and are presumed to be involved in the regulation of purine biosynthesis (Rappu et al. 1999).

ORF3 starts 61 nucleotides downstream of the *queD* stop codon, and a putative ribosome binding site (GGAG) was identified eight nucleotides upstream of the alternative start codon GTG. The 30,200 Da hypothetical protein (281 aa) exhibits 42% identity to amidohydrolase two from *Arthrobacter* sp. FB24 (acc no ZP_00414293). A conserved domain search revealed that the region spanning amino acids 9–195 is related to the amidohydrolase two protein family (Pfam signature PF04909).

Potential ORF4, with a possible Shine Dalgarno sequence (GAGG) 6 nucleotides upstream of its start codon, starts 89 nucleotides downstream of ORF3. The putative protein fragment (146 aa) resembles the N-terminal segment of dienelactone hydrolase (323 aa) from *Novosphingobium aromaticivorans* DSM 12444 with 41% identity, and the N-terminal segments of several esterases, e.g., carboxylesterase EST2 (310 aa) from *Alicyclobacillus acidocaldarius* (acc no 1EVQ_A, Manco et al. 1999), showing 35% identity within the aligned region. The amino acid segment comprising positions 60–139 of the ORF4 protein aligns with the N-terminal part of a carboxylesterase domain (Pfam signature PF00135). Carboxylesterases and dienelactone hydrolases are members of the α/β-hydrolase fold clan (Nardini and Dijkstra 1999).

FramePlot analysis of the 983 bp DNA sequence between putative ORF2 and *queD* showed that two regions exhibit a relatively high G + C content, suggesting two short open reading frames, starting 900 and 563 bp upstream of *queD*, which may code for hypothetical proteins of 144 and 188 aa. However, comparison of the deduced hypothetical proteins with the NCBI database did not reveal any significant matches, and it is not clear whether these ORFs indeed represent functional genes.

The quercetinase gene *queD* consists of 558 bp, encoding a protein of 186 amino acids with a calculated molecular mass of 21,065 Da. As described for other genes of Streptomycetes (Wright and Bibb 1992), *queD* exhibits a high overall G + C content (67.6%), with pronounced preference of G and C at the third codon position (93.6% G + C). Six nucleotides upstream of the initiation codon ATG, a possible ribosome binding site was identified (GGAGG). Analysis of the deduced amino acid sequence with SignalP and TatP did not predict a signal peptide, indicating that *Streptomyces* quercetinase in contrast to the fungal enzymes (Oka et al. 1971; Hund et al. 1999; Fusetti et al. 2002) is a cytoplasmic protein, as already obvious by its preparation from cell extracts of strain FLA as a soluble protein. *Streptomyces* QueD is most closely related to quercetinase of *Bacillus subtilis* (QueD, or YxaG, acc no P42106), with 35.9 and 29.0% identity of *Streptomyces* QueD to the C- and N-terminal domain of *Bacillus* QueD, respectively. Secondary structure predictions suggested that the order of α-helices and β-strands of QueD from strain FLA is similar to that of the C-terminal cupin domain of *Bacillus* quercetinase, indicating that the *Streptomyces* protein also exhibits the typical cupin β-barrel fold. The N- and C-terminal domains of quercetinase from *Aspergillus japonicus* (acc no Q7SIC2) exhibit 27.9 and 23.4% identity, respectively, to QueD from strain FLA. In a multiple comparison performed with ClustalW, the *Streptomyces* protein aligns with the C-terminal cupin domains of both *Bacillus* and *Aspergillus* quercetinase (Fig. 2). The alignment and secondary structure predictions show that, in contrast to the quercetinases of *B. subtilis* and *A. japonicus* which are both bicupins, *Streptomyces* QueD belongs to the monocupins.

The sequences ^{63}GEVIPAHSHADTYEVFYITQG^{83} and ^{104}GDFGFVPKNCVHAYRM^{119} of QueD from strain FLA match the consensus motifs G(X)$_5$HXH(X)$_{3,4}$E(X)$_6$G and G(X)$_5$PXG(X)$_2$H(X)$_3$N of the cupin superfamily (Dunwell et al. 2000, 2001, 2004). In the enzymatic members of this superfamily, the two histidine residues and the glutamate in motif 1 as well as histidine in motif 2 act as ligands for an active-site

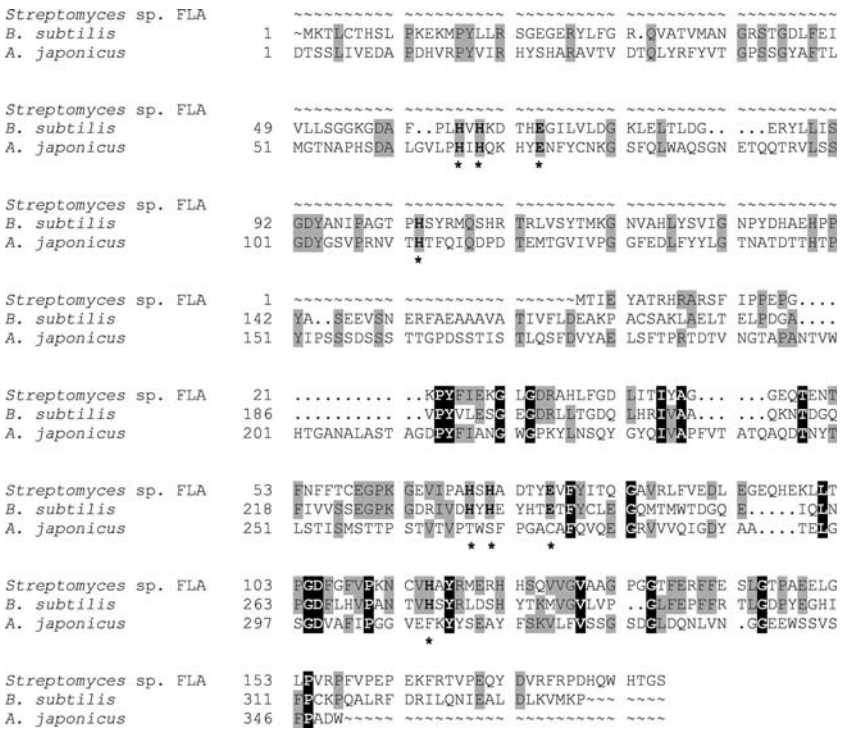


Fig. 2 Alignment of quercetinases from *Streptomyces* sp. FLA, *Bacillus subtilis* (acc no P42106) and *Aspergillus japonicus* (acc no Q7SIC2), compiled with the ClustalW program. Amino acids identical in two and three enzymes are highlighted in *gray* and *black*, respectively. Each catalytically active cupin domain comprises three conserved histidine residues and a glutamate (marked by *asterisks*), which act as ligands of the active site metal

divalent metal ion. Conservation of these residues suggests that QueD contains a catalytically relevant metal cofactor as well.

Heterologous expression of *queD* in *Streptomyces lividans* TK23

To confirm that the DNA fragment harbouring the *queD* gene confers quercetinase activity, the 3.4 kb *Bam*HI fragment (Fig. 1) was ligated into the *Streptomyces* vector pIJ702, resulting in pIJ702HM. *Streptomyces lividans* strain TK23, which does not exhibit quercetinase activity, was used as a host for the recombinant plasmid. Transformants formed zones of decolorisation on quercetin agar plates (Fig. 3), indicating formation of functional quercetinase in *S. lividans*. Crude extract of *S. lividans* TK23 pIJ702HM exhibited a specific quercetinase activity of 5.13 U mg^{-1}, which is about 26-fold higher than measured in extracts of *Streptomyces* sp. FLA. Such increase of specific activity in the recombinant strain is consistent with the approximate copy number of the cloning vector.

Overexpression of *queD* in *E. coli*, and properties of recombinant QueD His_6

The gene *queD* of *Streptomyces* sp. FLA was overexpressed in *E. coli* BL21 (DE3) pLysS pET23a*queD*, and the recombinant QueDHis_6 protein (calculated molecular mass of 23,041 Da) was purified by Ni^{2+} che-

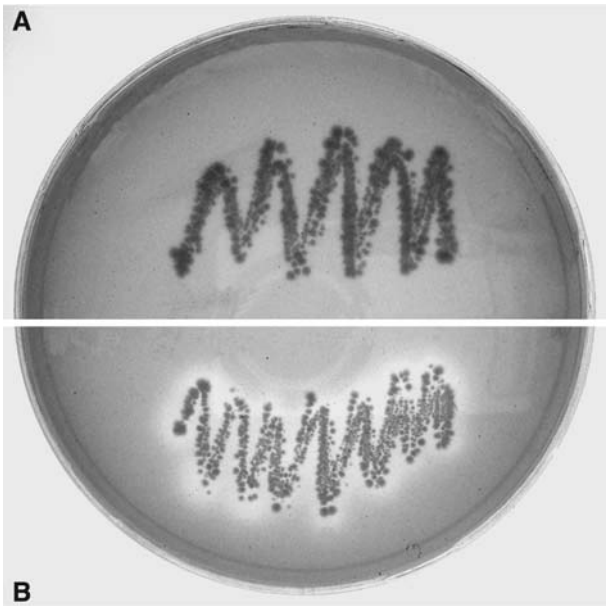


Fig. 3 *Streptomyces lividans* strain TK23 transformed with pIJ702 (**a**) and pIJ702HM (**b**) on mineral medium containing 2 mM quercetin and 0.5% glucose as carbon sources. Quercetinase activity is indicated by formation of colorless zones on the yellow quercetin agar

late affinity chromatography to near electrophoretic homogeneity (Fig. 4). About 30 mg of QueDHis_6 were obtained from 4.8 g of cells (wet biomass). The enrichment factor from crude extract supernatant was approximately 21, reflecting the high content of recombinant protein in the cell extract (Fig. 4). Gel filtration

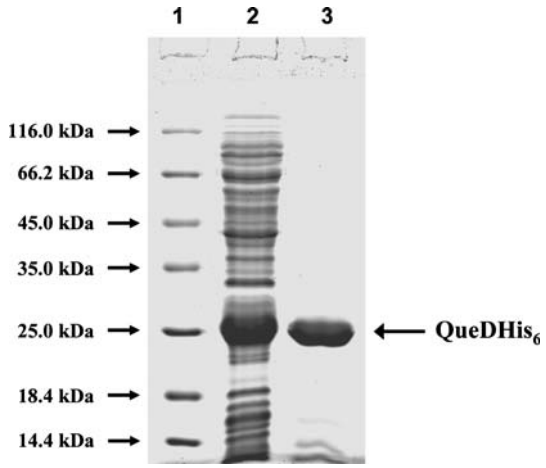


Fig. 4 SDS-PAGE of recombinant QueDHis$_6$. *Lane 1* Molecular mass standards; *lane 2* crude extract supernatant of *E. coli* BL21 (DE3) pLysS pET23a*queD*, obtained from cells harvested 6 h after IPTG induction; *lane 3* recombinant QueDHis$_6$ after purification by Ni^{2+} chelate affinity chromatography

of the purified protein indicated a molecular mass of approximately 63 kDa, suggesting that QueDHis$_6$ is a multimeric protein in its native state. Recombinant QueDHis$_6$ catalyzed the cleavage of quercetin with a specific activity of 3.96 U mg^{-1} (at pH 8) with concomitant release of CO, indicating that the gene product of *queD* is sufficient for quercetinase activity. From a V_{max} of 10.0 ± 0.77 µM min^{-1}, an apparent k_{cat} of 1.45 ± 0.11 s^{-1} was calculated for recombinant QueD His$_6$. This k_{cat} value is slightly higher than that of (likewise His-tagged) *B. subtilis* quercetinase, as isolated from recombinant *E. coli* cells grown in LB (0.8 s^{-1}; Bowater et al. 2004). The apparent K_m value of QueDHis$_6$ for quercetin, determined in the standard assay (pH 8) in air-saturated buffer, was 14.1 ± 0.7 µM. This value is higher than the K_m values reported for the quercetinases from *A. flavus* and *A. niger* of 5.2 µM (Oka et al. 1971) and 6.6 µM (Hund et al. 1999), respectively. *B. subtilis* quercetinase prepared from LB-grown recombinant *E. coli* also showed a low K_m of 3.8 µM (Bowater et al. 2004).

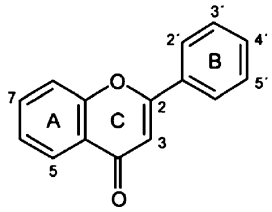
Several flavonoids were tested for their decomposition by recombinant *Streptomyces* quercetinase (Table 2). Besides quercetin, the flavonols kaempferol, myricetin, galangin, fisetin, and morin were converted by the enzyme. Kaempferol, which compared to quercetin lacks the 3′-OH group at the B-ring, was cleaved with relatively high rate, indicating that the substituent at this position of the benzene ring is not of major importance for catalysis. On the other hand, a hydroxy substituent at position 2′ appears to drastically impair the catalytic activity of QueDHis$_6$, since morin, which has a 2′,4′-dihydroxy substitution pattern at the B-ring, was converted with a relative rate of only 1.7%. This may be due to steric effects, and/or the particularly low pK_a (3.46) of one of its dissociating OH-groups (Jovanovic et al. 1994). In contrast, an additional hydroxy substituent at position C5′ of quercetin had a comparatively minor effect on catalysis by QueDHis$_6$, as indicated by its relative activity of about 50% towards myricetin (Table 2). Enzyme-catalyzed release of carbon monoxide was detected from all flavonols (3-hydroxy-flavones) tested, suggesting that they all undergo 2,4-dioxygenolytic ring cleavage (Table 2). An apparent relative activity of QueDHis$_6$ towards taxifolin of about 0.1% was measured by monitoring dioxygen consumption, but carbon monoxide formation was not observed. Since this flavonoid differs from the physiological substrate quercetin only by saturation of C2 and C3 of the *O*-heterocyclic ring, it is obvious that the C2–C3 double bond of the flavonols, which is conjugated with the 4-oxo group involved in electron delocalization, plays an important role in catalysis. Enzyme-catalyzed dioxygen consumption and CO formation were not observed when QueDHis$_6$ was incubated with the flavone luteolin, which apart from the missing hydroxyl group at C3 of the C-ring is identical to quercetin, demonstrating that the flavonol structure is indispensable for the dioxygenolytic ring cleavage reaction by QueDHis$_6$.

Discussion

In this study, the gene *queD* coding for CO-forming quercetinase was identified in *Streptomyces* sp. strain FLA, a soil isolate related to *Streptomyces eurythermus*^{T}. As indicated by hybridization of a *queD*-specific probe with genomic DNA of quercetin degrading *S. alboniger*, *S. eurythermus*, *S. flaveolus*, and *S. tendae* strains, the gene coding for quercetinase is fairly conserved among these Streptomycetes. Sequence analysis of DNA regions flanking *queD* suggests that the quercetinase gene in *Streptomyces* sp. strain FLA is not part of a catabolic gene cluster involved in the degradation of aromatic compounds. However, we can not exclude the possibility that the gene product of ORF4, which shows similarities to carboxylesterases, is an esterase catalyzing cleavage of 2-protocatechuoylphloroglucinol carboxylic acid, the product of the quercetinase reaction.

Bicupin proteins of the pirin family (pfam acc no PF02678), which bind Fe^{2+} (Pang et al. 2004), are highly conserved among both eukaryotes and prokaryotes, but their function is poorly understood. They

Table 2 Basic flavone structure and activity of recombinant QueDHis$_6$ towards flavonoids



Substrate	Synonym	Relative activity (%)	CO formation
Quercetin	3,5,7,3′,4′-pentahydroxy-flavone	100	+
Kaempferol	3,5,7,4′- tetrahydroxy-flavone	70	+
Myricetin	3,5,7,3′,4′,5′-hexahydroxy-flavone	49	+
Galangin	3,5,7-trihydroxy-flavone	28	+
Fisetin	3,7,3′,4′-tetrahydroxy-flavone	23	+
Morin	3,5,7,2′,4′-pentahydroxy-flavone	1.7	+
Taxifolin	3,5,7,3′,4′-pentahydroxy-2,3-dihydro-flavone	0.1	–
Luteolin	5,7,3′,4′-tetrahydroxy-flavone	ND[a]	–

[a] ND Not detected

have been proposed to be involved in co-regulation of transcription and DNA replication; however, recently it has been shown that human pirin and its *E. coli* homologue Yhhw possess quercetinase activity. Since quercetin interferes with various cellular pathways in both eukaryotes and prokaryotes, it has been discussed that the quercetinase activity of pirins might be important to counteract its inhibitory effects (Adams and Jia 2005). In enteric bacteria like *E. coli* as well as in soil bacteria like *Streptomyces* sp. FLA, which are very likely to encounter plant polyphenols in their natural habitat, quercetinase activity might indeed be most important for detoxification, since quercetin exhibits antimicrobial activities (for review, see Cushnie and Lamb 2005). It was reported to cause an increase in the permeability of the inner bacterial membrane and a dissipation of the membrane potential (Mirzoeva et al. 1997), to inhibit bacterial DNA gyrase B at its ATP binding site, and to induce DNA cleavage at the DNA-gyrase complex (Plaper et al. 2003). Bacterial quercetinases thus may play a primary role in detoxification, but additionally in some organisms, such as *Aspergillus* spp., these proteins may have evolved to potent catabolic enzymes. However, the observed marginal growth of *Streptomyces* sp. FLA on quercetin suggests that in this strain, quercetin metabolism is not optimized for using this compound as source of carbon and energy.

Amino acid sequences and crystal structures are available for *A. japonicus* and *B. subtilis* quercetinase. In the latter protein, the N- and C-terminal cupin domains both comprise a set of three strictly conserved histidines and a glutamate residue, each set coordinating a divalent metal ion, and both domains are catalytically active (Bowater et al. 2004; Gopal et al. 2005). In contrast to *Bacillus* quercetinase, the three potential ligands to a metal are missing in the C-terminal cupin domain of the *A. japonicus* enzyme (Fig. 2). *A. japonicus* quercetinase thus possesses only one active site in its N-terminal cupin domain, where H66, H68, H112, and a water molecule or E73 coordinate a Cu^{2+} ion (Fusetti et al. 2002). Dunwell et al. (2004) reported that the majority of bicupin dioxygenases have only one active domain, whilst the other is a non-functional remnant.

Since pirins as well as *Bacillus* and *Aspergillus* quercetinases are bicupins, the enzyme of *Streptomyces* sp. FLA is the only monocupin quercetinase described as yet, indicating that the interface between the two domains of the bicupin quercetinases is not essential for catalytic activity. Bicupins have been suggested to have evolved by gene duplication and then fusion from ancestral single cupin progenitors, or in some cases by fusion of two different monocupin precursor genes (Dunwell et al. 2000, 2001, 2004). Based on this hypothesis, the *Streptomyces* enzyme might represent an "ancient" bacterial quercetinase.

Purified recombinant QueDHis$_6$ exhibited a specific activity of about 4 U mg^{-1} and an apparent k_{cat} of only 1.45 s^{-1} towards quercetin. Such poor specific activity compared to wild-type quercetinase from strain FLA (98 U mg^{-1}) might be due to the cultivation of the *E. coli* clone in LB medium. *Bacillus* quercetinase also showed a low k_{cat} of 0.8 s^{-1} when purified from recombinant *E. coli* grown in LB (Bowater et al. 2004); under these conditions, the *Bacillus* enzyme was synthesized as an iron protein (Barney et al. 2004; Bowater et al. 2004).

Notably, the divalent metal ions present in the growth medium during gene expression significantly influence the metal content and catalytic activity of recombinant *Bacillus* quercetinase. Addition of Mn^{2+} and Co^{2+} to minimal medium (M9) resulted in highest quercetinase activity in cell extracts, and purified Mn-QueD and Co-QueD showed k_{cat} values of 25 and 6.7 s^{-1}, respectively (Schaab et al. 2006). The authors suggested that the low k_{cat} of quercetinase purified from LB-grown clones is due to the 20- to 30-fold lower intracellular concentration of manganese compared to iron in LB-grown *E. coli* (Gabbianelli et al. 1995), which controls Fe incorporation into the active site. Concerning *Streptomyces* quercetinase, it is conceivable that the LB medium likewise does not provide sufficient amounts of the metal required for optimal activity.

$QueDHis_6$ of *Streptomyces* sp. FLA was found to catalyze cleavage of the flavonols kaempferol, myricetin, galangin, fisetin, and morin. These compounds are also converted by quercetinase of *A. flavus*, and the relative activities of the two enzymes show similar tendencies, apart from the activity towards kaempferol, which in case of the fungal enzyme is 2.5-fold higher than towards quercetin (Oka et al. 1972). The failure of recombinant $QueDHis_6$ to decompose the flavone luteolin ("3-desoxyquercetin") and to catalyze CO release from the 2,3-dihydroflavonol taxifolin suggests that the flavonol scaffold is essential for the enzyme-catalyzed reaction. Such observation is consistent with the reaction mechanisms proposed for Cu^{2+}-quercetinase of *A. japonicus* (Steiner et al. 2002) and the Mn^{2+}-enzyme of *B. subtilis* (Schaab et al. 2006). Despite important mechanistic differences, both reactions involve quercetin binding to the metal centre via its O3 atom, and single-electron oxidation of the flavonol ring, in which the generated radical is stabilized by the delocalised pi-electron system.

The functionally highly variable cupin superfamily comprises several types of dioxygenases (Dunwell et al. 2000, 2001, 2004). Within the monocupin dioxygenases, the predominant subclass is that of the 2-oxoglutarate- and Fe^{2+}-dependent dioxygenases. Further monocupin dioxygenases are 3-hydroxyanthranilate 3,4-dioxygenase (EC 1.13.11.6), cysteine dioxygenase (EC 1.13.11.20), and acireductone dioxygenase (ARD) from *Klebsiella pneumoniae* (gi:75475369; PDB 1M4O). A most interesting feature of the latter protein is its ability to catalyze different reactions with Ni^{2+} or Fe^{2+} bound to its active site. Fe-ARD catalyzes a reaction that is part of the ubiquitous methionine salvage pathway, converting 1,2-dihydroxy-3-keto-5-(methylthio)pentene to formate and 2-keto-4-(methylthio)butanoate, the α-ketoacid precursor of methionine. In contrast, Ni-ARD yields methylthiopropanoate, CO and formate from the same substrate, i.e., it catalyzes the dioxygenolytic cleavage of two carbon–carbon bonds with concomitant release of CO, a reaction analogous to that of quercetinase. However, the in vivo significance of this reaction is unknown (Dai et al. 1999, 2001; Pochapsky et al. 2002). Ni-ARD-type activity was observed when the apoprotein was reconstituted with Mn^{2+}, Co^{2+}, and Ni^{2+}, whereas Fe-ARD activity was conferred by Fe^{2+} and Mg^{2+} (Pochapsky et al. 2002). ARD proteins constitute an own enzyme family (Pfam acc no PF03079); however, *Klebsiella* ARD and *Streptomyces* QueD share 21.7% identity of their amino acid sequences.

The cupin fold is well adapted for dioxygenases, since its signature motifs provide His and Glu ligands for binding a divalent metal ion that can be used for the activation of molecular oxygen, the organic substrate, or both. Among dioxygenases, iron is the most commonly used transition metal. Copper, which became biologically available later than iron, is found in a much smaller range of oxygenases (Sariaslani 1989; Harayama et al. 1992). Based on structural as well as mechanistic considerations, quercetinase of *A. japonicus* has been suggested to have evolved from a primordial Fe-containing enzyme (Fusetti et al. 2002; Steiner et al. 2002). The *Bacillus* enzyme, on the other hand, may be a member of the manganese dioxygenase family, and recent kinetic and spectroscopic evidence suggests that the catalytic mechanisms of *Aspergillus* and *Bacillus* quercetinases are different (Schaab et al. 2006). Identification of the metal cofactor of *Streptomyces* quercetinase may contribute further insight into the evolutionary relationship of bacterial and fungal quercetinases and into the versatility of these enzymes in terms of their metal cofactor and catalytic mechanism.

Acknowledgments We thank Prof. Dr. H. Pape, Münster, for kindly providing the *Streptomyces* sp. strains (except strain FLA). The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged (FE 383/9-1).

References

Adams M, Jia Z (2005) Structural and biochemical analysis reveal pirins to possess quercetinase activity. J Biol Chem 280:28675–28682

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Arendt R, Dörmer L (1972) Technik der Experimentalchemie, 9th edn. Quelle & Meyer, Heidelberg

Barney BM, Schaab MR, LoBrutto R, Francisco WA (2004) Evidence for a new metal in a known active site: purification and characterization of an iron-containing quercetin 2,3-dioxygenase from *Bacillus subtilis*. Protein Expr Purif 35:131–141

Barz W (1971) Über den Abbau aromatischer Verbindungen durch *Fusarium oxysporum* Schlecht. Arch Mikrobiol 78:341–352

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: signalP 3.0. J Mol Biol 340:783–795

Bendtsen JD, Nielsen H, Widdick D, Palmer T, Brunak S (2005) Prediction of twin-arginine signal peptides. BMC bioinformatics 6:167

Bowater L, Fairhurst SA, Just VJ, Bornemann S (2004) *Bacillus subtilis* YxaG is a novel Fe-containing quercetin 2,3-dioxygenase. FEBS Lett 557:45–48

Butler MJ, Binnie C, DiZonno MA, Krygsman P, Soltes GA, Soostmeyer G, Walczyk E, Malek LT (1995) Cloning and characterization of a gene encoding a secreted tripeptidyl aminopeptidase from *Streptomyces lividans* 66. Appl Environ Microbiol 61:3145–3150

Cushnie TP, Lamb AJ (2005) Antimicrobial activity of flavonoids. Int J Antimicrob Agents 26:343–356

Dai Y, Wensink PC, Abeles RH (1999) One protein, two enzymes. J Biol Chem 274:1193–1195

Dai Y, Pochapsky TC, Abeles RH (2001) Mechanistic studies of two dioxygenases in the methionine salvage pathway of *Klebsiella pneumoniae*. Biochemistry 40:6379–6387

Dunwell JM, Khuri S, Gane PJ (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. Microbiol Mol Biol Rev 64:153–179

Dunwell JM, Culham A, Carter CE, Sosa-Aguirre CR, Goodenough PW (2001) Evolution of functional diversity in the cupin superfamily. Trends Biochem Sci 26:740–746

Dunwell JM, Purvis A, Khuri S (2004) Cupins: the most functionally diverse protein superfamily? Phytochemistry 65:7–17

Duthie GG, Gardner PT, Kyle JAM (2003) Plant polyphenols: are they the new magic bullet? Proc Nutr Soc 62:599–603

Fusetti F, Schröter KH, Steiner RA, van Noort PI, Pijning T, Rozeboom HJ, Kalk KH, Egmond MR, Dijkstra BW (2002) Crystal structure of the copper-containing quercetin 2,3-dioxygenase from *Aspergillus japonicus*. Structure 10:259–268

Gabbianelli R, Battistoni A, Polizio F, Carrì MT, De Martino A, Meier B, Desideri A, Rotilio G (1995) Metal uptake of recombinant cambialistic superoxide dismutase from *Propionibacterium shermanii* is affected by growth conditions of host *Escherichia coli* cells. Biochem Biophys Res Commun 216:841–847

Gopal B, Madan LL, Betz SF, Kossiakoff AA (2005) The crystal structure of a quercetin 2,3-dioxygenase from *Bacillus subtilis* suggests modulation of enzyme activity by a change in the metal ion at the active site(s). Biochemistry 44:193–201

Grant SGN, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc Natl Acad Sci USA 87:4645–4649

Hames BD (1990) One-dimensional polyacrylamide gel electrophoresis. In: Hames BD, Rickwood D (eds) Gel electrophoresis of proteins—a practical approach, vol 2, IRL Press, Oxford University Press, pp 1–147

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580

Hanes CS (1932) Studies on plant amylase. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. Biochem J 26:1406–1421

Harayama S, Kok M, Neidle EL (1992) Functional and evolutionary relationships among diverse oxygenases. Annu Rev Microbiol 46:565–601

Herrmann AP, Willems M, Janke HD (2001) Degradation of natural polyphenols by methanogenic consortia enriched from digested municipal sludge. Water Res 35:2575–2582

Higgins DG, Thompson JD, Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. Meth Enzymol 266:383–402

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM, Schrempf H (1985) Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Foundation, Norwich

Hosny M, Dhar K, Rosazza JPN (2001) Hydroxylations and methylations of quercetin, fisetin, and catechin by *Streptomyces griseus*. J Nat Prod 64:462–465

Hund HK, Breuer J, Lingens F, Hüttermann J, Kappl R, Fetzner S (1999) Flavonol 2,4-dioxygenase from *Aspergillus niger* DSM 821, a type 2 Cu^{II}-containing glycoprotein. Eur J Biochem 263:871–878

Ishikawa J, Hotta K (1999) FramePlot: a new implementation of the Frame analysis for predicting protein-coding regions in bacterial DNA with a high G + C content. FEMS Microbiol Lett 174:251–253

Iwashina T (2000) The structure and distribution of the flavonoids in plants. J Plant Res 113:287–299

Jovanovic SV, Steenken S, Tosic M, Marjanovic B, Simic MG (1994) Flavonoids as antioxidants. J Am Chem Soc 116:4846–4851

Katz E, Thompson CJ, Hopwood DA (1983) Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J Gen Microbiol 129:2703–2714

Kieser T, Mervyn JB, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. John Innes Foundation, Norwich

Kim BG, Jung BR, Lee Y, Hur HG, Lim Y, Ahn JH (2006) Regiospecific flavonoid 7-*O*-methylation with *Streptomyces avermitilis O*-methyltransferase expressed in *Escherichia coli*. J Agric Food Chem 54:823–828

Krieger TJ, Bartfeld D, Jenish DL, Hadary D (1994) Purification and characterization of a novel tripeptidylaminopeptidase from *Streptomyces lividans* 66. FEBS Lett 352:385–388

Kooter IM, Steiner RA, Dijkstra BW, van Noort PI, Egmond MR, Huber M (2002) EPR characterization of the mononuclear Cu-containing *Aspergillus japonicus* quercetin 2,3-dioxygenase reveals dramatic changes upon anaerobic binding of substrates. Eur J Biochem 269:2971–2979

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lamson DW, Brignall MS (2000) Antioxidants and cancer III: quercetin. Altern Med Rev 5:196–208

Locci R (1989) Streptomycetes and related genera. In: Williams ST, Sharpe ME, Holt JG (eds) Bergey´s manual of systematic bacteriology, vol 4, Williams and Wilkins, Baltimore, pp 2451–2508

Manco G, Febbraio F, Adinolfi E, Rossi M (1999) Homology modeling and active-site residues probing of the thermophilic *Alicyclobacillus acidocaldarius* esterase 2. Protein Sci 8:1789–1796

Marchler-Bauer A, Anderson JB, Cherukuri PF, DeWeese-Scott C, Geer LY, Gwadz M , He S, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Liebert CA, Liu C, Lu F, Marchler GH, Mullokandov M, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Yamashita RA, Yin JJ, Bryant SH (2005) CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res 33:192–196

Mirzoeva OK, Grishanin RN, Calder PC (1997) Antimicrobial action of propolis and some of its components: the effects on

growth, membrane potential and motility of bacteria. Microbiol Res 152:239–246
Morishita R, Kawagoshi A, Sawasaki T, Madin K, Ogasawara T, Oka T, Endo Y (1999) Ribonuclease activity of rat liver perchloric acid-soluble protein, a potent inhibitor of protein synthesis. J Biol Chem 274:20688–20692
Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164:165–172
Nardini M, Dijkstra BW (1999) Alpha/beta hydrolase fold enzymes: the family keeps growing. Curr Opin Struct Biol 9:732–737
Needleman SB, Wunsch CD (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J Mol Biol 48:443–453
Oka T, Simpson FJ, Child JJ, Mills C (1971) Degradation of rutin by *Aspergillus flavus*. Purification of the dioxygenase, quercetinase. Can J Microbiol 17:111–118
Oka T, Simpson FJ, Krishnamurty HG (1972) Degradation of rutin by *Aspergillus flavus*. Studies on specifity, inhibition, and possible reaction mechanism of quercetinase. Can J Microbiol 18:493–508
Pang H, Bartlam M, Zeng Q, Miyatake H, Hisano T, Miki K, Wong L-L, Gao GF, Rao Z (2004) Crystal structure of human pirin. An iron-binding nuclear protein and transcription cofactor. J Biol Chem 279:1491–1498
Pietta PG (2000) Flavonoids as antioxidants. J Nat Prod 63:1035–1042
Plaper A, Golob M, Hafner I, Oblak M, Šolmajer T, Jerala R (2003) Characterization of quercetin binding site on DNA gyrase. Biochem Biophys Res Commun 306:530–536
Pochapsky TC, Pochapsky SS, Ju T, Mo H, Al-Mjeni F, Maroney MJ (2002) Modeling and experiment yields the structure of acireductone dioxygenase from *Klebsiella pneumoniae*. Nat Struct Biol 9:966–972
Pospiech A, Neumann B (1995) A versatile quick-prep of genomic DNA from Gram-positve bacteria. Trends Genet 11:217–218
Prior RL (2003) Fruits and vegetables in the prevention of cellular oxidative damage. Am J Clin Nutr 78:570–578
Rao JR, Cooper JE (1994) Rhizobia catabolize *nod* gene-inducing flavonoids via C-ring fission mechanisms. J Bacteriol 176:5409–5413
Rappu P, Shin BS, Zalkin H, Mantsala P (1999) A role for a highly conserved protein of unknown function in regulation of *Bacillus subtilis* purA by the purine repressor. J Bacteriol 181:3810–3815
Roche molecular biochemicals (1995) The DIG System User´s Guide for Filter Hybridization (ISBN 3-88630-200-8) Boehringer Mannheim GmbH, Mannheim
Rose K, Fetzner S (2006) Identification of linear plasmid pAM1 in the flavonoid degrading strain *Actinoplanes missouriensis*^{T} (DSM 43046). Plasmid 55:249–254
Rost B, Yachdav G, Liu J (2004) The PredictProtein server. Nucleic Acids Res 32:W321–326
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
Sariaslani FS (1989) Microbial enzymes for oxidation of organic molecules. Crit Rev Biotechnol 9:171–257
Schaab MR, Barney BM, Francisco WA (2006) Kinetic and spectroscopic studies on the quercetin 2,3-dioxygenase from *Bacillus subtilis*. Biochemistry 45:1009–1016
Schlegel HG, Kaltwasser H, Gottschalk G (1961) A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies. Arch Microbiol 38:209–222
Schneider H, Blaut M (2000) Anaerobic degradation of flavonoids by *Eubacterium ramulus*. Arch Mikrobiol 173:71–75
Schultz E, Engle FE, Wood JM (1974) New oxygenases in the degradation of flavones and flavanones by *Pseudomonas putida*. Biochemistry 13:1768–1776
Steiner RA, Kalk KH, Dijkstra BW (2002) Anaerobic enzyme·substrate structures provide insight into the reaction mechanism of the copper-dependent quercetin 2,3-dioxygenase. Proc Natl Acad Sci USA 99:16625–16630
Sutherland JB, Crawford DL, Pometto AL 3rd (1983) Metabolism of cinnamic, *p*-coumaric and ferulic acids by *Streptomyces setonii*. Can J Microbiol 29:1253–1257
Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Biochemistry 76:4350–4354
Vieira J, Messing J (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268
Waterman MR (1978) Spectral characterization of human hemoglobin and its derivates. Meth Enzymol 52:456–463
Westlake DWS, Talbot G, Blakley ER, Simpson FJ (1959) Microbial decomposition of rutin. Can J Microbiol 5:621–629
Westlake DWS, Roxburgh JM, Talbot G (1961) Microbial production of carbon monoxide from flavonoids. Nature 189:510–511
Winter J, Moore LH, Dowell JR.VR, Bokkenheuser (1989) C-ring cleavage of flavonoids by human intestinal bacteria. Appl Environ Microbiol 55:1203–1208
Wright F, Bibb MJ (1992) Codon usage in the G + C-rich *Streptomyces* genome. Gene 113:55–65
Yoon Y, Yi YS, Lee Y, Kim S, Kim BG, Ahn JH, Lim Y (2005) Characterization of *O*-methyltransferase ScOMT1 cloned from *Streptomyces coelicolor* A3(2). Biochim Biophys Acta 1730:85–95
Zor T, Selinger Z (1996) Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal Biochem 236:302–308