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Biotransformation of adenine and cytokinins by the rhizobacterium *Serratia proteamaculans*

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Abstract

Approximately 60,000 microorganisms from Saskatchewan soil were screened for growth on the cytokinin N^6-benzyladenine (BA) as C source. A single isolate, identified as *Serratia proteamaculans*, grew well on BA. The culture filtrates from *S. proteamaculans* were screened using reversed phase high performance liquid chromatography (RP-HPLC) for the presence of secondary metabolites. The analysis revealed a major metabolite and its chemical structure was deduced as 8-hydroxy-N^6-benzyladenine (8-OHBA). Subsequently, the *S. proteamaculans* isolate was also found to metabolize N^6-(2-isopentenyl)adenine and adenine through oxidation of C-8 of the purine ring. A clone of the *S. proteamaculans* xanthine dehydrogenase (Xdh, EC 1.1.1.204) encoding genes was isolated in *Escherichia coli*. This *E. coli* isolate metabolized BA to 8-OHBA. Similar to other bacterial Xdh, the *S. proteamaculans* enzyme was composed of two subunits. The derived amino acid sequences of these Xdh subunits were most similar (XdhA, 60%; XdhB, 72%) to those of *Pseudomonas aeruginosa*.

Keywords: 6-Benzylaminopurine; 6-(γ,γ-Dimethylallylamino)purine; Plant growth promoting rhizobacteria; PGPR; Phytohormone

1. Introduction

Plant growth promotion by rhizobacteria (PGPR) requires, firstly, the proliferation of the bacterium in the rhizosphere of the host (Bull et al., 1991). That proliferation is dependent on the ability of the microorganism to utilize, specifically, root exudate compounds as a carbon (C) source (Campbell et al., 1997; Goddard et al., 2001). Further, compounds present in these exudates vary among plant species and thus can provide selective nourishment. For example, among leguminous plants, only pea (*Pisum sativa* L.) roots exude the amino acid homoserine. The pea-nodulating bacterium *Rhizobium leguminosarum* grows well on homoserine as sole C and nitrogen (N) source but *R. trifolii*, *R. phaseoli* and *R. meliloti* that nodulate other legumes show little to no growth on this substrate (van Egeraat, 1975). Phytohormones including auxins, cytokinins, gibberellins, ethylene and abscisic acid are among the more unusual compounds present in the rhizosphere versus bulk soil (reviewed in Frankenberger and Arshad, 1995). As such, microorganisms that can metabolize phytohormones may be able to better proliferate in the rhizosphere than organisms that lack the ability. The aim of this study was the isolation and characterization of microorganisms that metabolize cytokinins for use in an investigation of PGPR competence.

2. Results and discussion

Approximately 60,000 colony-forming units (cfu) were plated onto M9 minimal medium containing N^6-benzyladenine (BA) as the carbon source. After one-week incubation, small colonies were found growing on the plates. The colonies were streaked onto the selective medium twice more to isolate the bacterium. A single isolate designated

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B1 continued to show good growth on the selective medium. The identity of the B1 isolate was determined by database comparison of the 5′ and 3′ sequences of a PCR product from the gene for the 16S ribosomal RNA (rRNA) (DQ417493 and DQ417494). Basic local alignment search tool (BLAST) analysis of the partial sequence of the PCR product revealed greater than 99% similarity to sequence entries for *Serratia proteamaculans* 16S rRNA. Species of the genus *Serratia* are frequently found in the rhizosphere and several of the species are classified as PGPR. It appears that the primary growth promotion activity of these species is due to the production of compounds or enzymes that are antagonistic to plant pathogen survival (Kamensky et al., 2003). Additionally, most species produce indole acetic acid or closely related compounds in culture (Kalbe et al., 1996).

The crude culture filtrates from *S. proteamaculans* B1 incubated with benzyladenine (**1**, Fig. 1A) were screened using RP-HPLC for the presence of potential metabolites. The analysis revealed a major polar metabolite with $RR_t = 9.8$ min and several minor peaks. The metabolite was isolated and its chemical structure was deduced as **2** from comparison of its spectroscopic data with that of **1**.

The UV spectrum of metabolite **2** showed two absorption maxima at 213 and 276 nm, similar to that of **1**. The spectrum of **2** from high-resolution mass spectrometry using electron ionization detection mode (HREIMS) exhibited a molecular ion of 241 mass units in good agreement with the molecular formula of $C_{12}H_{11}N_5O$. This suggested that relative to **1** ($C_{12}H_{11}N_5$), compound **2** contained an additional oxygen atom. The ^{1}H NMR spectrum of **2** (Table 1) showed the same features as the ^{1}H spectrum of **1**: a doublet ($J = 5.5$ Hz) at δ 4.63 ppm, three signals (δ 7.31–7.35 ppm) corresponding to an aromatic ring, one singlet at δ 8.03 ppm, and three exchangeable protons at δ 6.84, 9.83 and 11.29 ppm, respectively. Decoupling experiments assigned the signal at δ 6.84 ppm to N^{10}–H, while δ 11.29 ppm was assigned to N^9–H, which was also the most downfield proton in ^{1}H NMR spectrum of **1**. The additional exchangeable proton at δ 9.83 ppm could be due to either an N–H or O–H. For **1**, the signals for H-2 and H-8 correspond to the singlets with δ 8.16 and 8.10 ppm, respectively. The ^{1}H NMR spectrum of **2** indicated that the signal for one of these protons was missing, suggesting that the oxygen atom was present at either C-2 or C-8. However, the closeness of the chemical shift δ 8.03 to both mentioned values meant that the position of the oxygen atom could not be unambiguously assigned. The benzyl moiety appeared to be intact, as also suggested by ^{13}C NMR spectroscopic data. That the oxygen atom was attached to C-8 rather than C-2 was finally deduced from analysis of the heteronuclear multiple-bond connectivity (HMBC) spectroscopic data. Thus, both methylene protons and the N^{10}–H showed a HMBC correlation with C-6 (δ 145.4 ppm). A correlation with C-6 was also observed from the C-H from the purine ring (δ 8.03 ppm). Since this had to be H-2 for the correlation to be observed, it was concluded that the oxygen atom was present at C-8. Moreover, the nuclear Overhauser enhancement (nOe) spectroscopy experiment showed a correlation between N^9–H and O–H, which strongly suggested the existence of this compound as an enol rather than an 8-keto tautomer.

Additional evidence for the presence of the oxygen atom at C-8 of the purine ring was gained by the hydrogenolysis of the benzyl group of metabolite **2** which yielded 8-hydroxyadenine (**3**), as shown in Fig. 1A. The ^{1}H NMR and MS data for this compound was in good agreement with those reported (Lindsay et al., 1999). In the literature however, the prefix '8-oxo' was used rather than '8-hydroxy' in naming this class of compounds, based on the research by Cho and Evans (1991) on 8-hydroxyadenosine. Moreover, the presence of the oxygen atom at C-2

Table 1
^{1}H and ^{13}C NMR spectroscopic assignments for 8-hydroxy-6-benzyladenine (**2**) in $(CD_3)_2SO$

Carbon/ atom #	^{13}C[a] δ (ppm) (*mult*)	^{1}H δ (ppm), multiplicity (J in Hz)	HMBC
2	150.8 (*d*)	8.03 (*s*, 1H)	C-4, C-6, C-8
4	147.4 (*s*)		
5	104.5 (*s*)		
6	145.4 (*s*)		
8	152.6 (*s*)		
9 NH		11.29 (*s*, 1H)	C-5, C-8
10 NH		6.84 (*m*, 1H)	C-5, C-6, C-11
11	43.5 (t)	4.63 (*d*, $J = 5.5$ Hz, 2H)	C-6, C-12, C-13
12	139.5 (*s*)		
13	127.5 (*d*, 2×)	7.31–7.35 (*m*, 2H)	C-11, C-12
14	128.4 (*d*, 2×)	7.31–7.35 (*m*, 2H)	C-13
15	127.0 (*d*)	7.25 (*m*, 1H)	
16 OH		9.83 (*br s*, 1H)	C-4, C-5, C-8

[a] Multiplicities were established from the HMQC correlations.

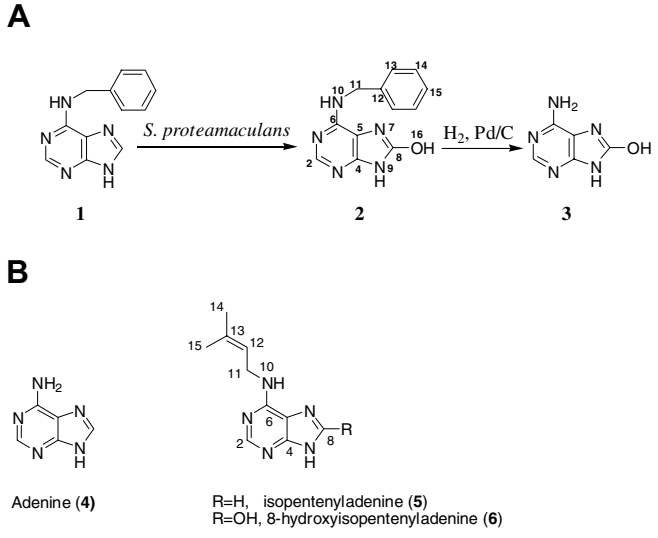

Fig. 1. (A) Biotransformation of N^6-benzyladenine (**1**) by *Serratia proteamaculans* to 8-hydroxy-N^6-benzyladenine (**2**) and its chemical degradation to 8-hydroxyadenine (**3**). (B) Chemical structures of adenine (**4**), isopentenyladenine (**5**) and 8-hydroxyisopentenyladenine (**6**).

Table 2
^{1}H and ^{13}C NMR spectroscopic assignments for 8-hydroxyisopentenyladenine (**6**) in $(CD_3)_2SO$

Carbon/ atom #	^{13}C[a] δ (ppm) (*mult*)	^{1}H δ (ppm) multiplicity (J in Hz)	HMBC
2	150.8 (*d*)	8.02 (*s*, 1H)	C-4, C-6
4	147.1 (*s*)		
5	104.4 (*s*)		
6	145.4 (*s*)		
8	152.5 (*s*)		
9 NH		11.21 (*s*, 1H)	
10 NH		6.40 (t, $J = 5.8$ Hz, 1H)	C-5, C-6, C-12
11	37.8 (*t*)	3.97 (d, $J = 5.8$ Hz, 2H)	C-6, C-12, C-13
12	121.4 (*d*)	5.29 (t, $J = 6.9$ Hz, 1H)	C-11, C-14, C-15
13	134.6 (*s*)		
14	25.3 (*sept*)	1.70 (s, 3H)	C-12, C-13
15	17.7 (*sept*)	1.68 (s, 3H)	C-12, C-13
16 OH		9.81 (*br s*, 1H)	

[a] Multiplicities were established from the HMQC correlations.

was also ruled out since N^6-benzyl-2-hydroxyadenine was synthetically prepared from N^6-benzyladenine-3-oxide (Itaya et al., 1999) and its spectroscopic data did not match that of metabolite **2**.

A similar biotransformation pattern was observed when adenine (**4**) or N^6-(2-isopentenyl)adenine (2iP) (**5**) were used as the major carbon source (Fig. 1B). As expected, isolate B1 metabolized these substrates through oxidation at C-8 of the purine ring, giving 8-hydroxyadenine (**3**) and 8-hydroxyisopentenyladenine (**6**) as biotransformation products. Their structure was confirmed either through comparison with the literature spectroscopic data (**3**) or was deduced from comparison with MS and NMR spectroscopic data for the starting material (**6**, McLennan and Pater, 1973; Chen and Cyr, 1982). We present the ^{1}H and ^{13}C NMR spectroscopic assignments for 8-hydroxyisopentenyladenine (**6**) in Table 2.

The C8-oxidation of purine bases is one of the major forms of oxidative base damage and can result from ionizing radiation and other oxygen radical generating systems. 8-Hydroxyadenine (**3**) has been detected in neoplastic liver of fish, in urine samples of ferrets and humans (Cho and Evans, 1991 and references therein) and in the ascidian *Symplegma rubra* (Lindsay et al., 1999). Additionally, Kang and Fenical (1997) isolated a unique zwitterionic benzyl substituted hydroxyadenine, aplidiamine, from the ascidian *Aplidiopsis* sp. However, a bacterial consortium of *Klebsiella* and *Rhodococcus* strains was unable to carry out the C8-oxidation of adenine except in the presence of *N*-methylmaleimide (Madyastha and Sridhar, 1999).

More specifically related to oxidation of cytokinins, Chen et al. (1975) were able to synthesize the 8-hydroxy and 2,8-dihydroxy derivatives of zeatin and 2iP in a cell free system using xanthine oxidase from milk. Therefore,

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XdhA At   1 MKDAISFILNSETISLKDFGPTDTLLDYLRISKRLTGTKEGCAEGDCGACTVLVGRLL-----DGSLRYESVNACIRFLG
XdhA Rc   1 --MEIAFLLNGETRRVRIEDPTQSLLELLRAEG-LTGTKEGCNEGDCGACTVMI-R--------DAAGSRAVNACLMMLP
XdhA Pa   1 ---MIRFLLNREIRVEERLDPNLTVLDYLRRHLGKTGTKEGCASGDCGACTVVVGELVTGEDGAERIRYRSLNSCLTFVS
XdhA Sp   1 ---MIQFLLNQTLKTETALDPNTTVLNYLRRDLGRCGTKEGCASGDCGACTVVLAEPE-----GDRLRYRSVNACLTFVS
                * **** *       **  * * ***      ******* ********              ********* *
             76 SLHGTHIVTVEHLAVRDGALHPVQQAMVDFHGSQCGFCTPGFIMSLYGLWLSSETPGRADIEKALQGNLCRCTGYEPIVR
             69 QIAGKALRTIEGIAAPDGRLHPVQQAMIDHHGSQCGFCTPGFIVSMA----AAHDRDRKDYDDLLAGNLCRCTGYAPILR
             78 ALHGKQLITVEDLKH-QDRLHDVQQAMVDCHGSQCGFCTPGFVMSLFALQKNSAGADPAKAHEALAGNLCRCTGYRPILD
             73 ALHGKQLITVEDLKQ-QGELHGVQQAMVDCHGSQCGFCTPGFVMSMFCLQKNTASYQREQAQQALAGNLCRCTGYRPILA
                 **** * *** *    **** ******* ************ **   *           *********** ***
            156 AAEKIAAARPSALFDPLQRDRTDIMAKLWAVRENETIIVTHGEDRSIIPATLSDLTEIYAAEPKATIVAGSTDVGLWVTK
            145 AAEAAAGE-PP--ADWLQADAAFTLAQLSSG-------VRGQTAPAFLPETSDALADWYLAHPEATLIAGGTDVSLWVTK
            157 AAEQACCHKRA---DQFDAREAATIEQLRAIAPRETAELNSGDRRCLLPLTVADLADLYGANPQARLLAGGTDLALEVTQ
            152 AAQQACDNPQP---DSFDHHQPQTLQRLQAIR--AATPLEAEGKRCLLPQSLDELARLYQQHPQAKLLAGGTDLALEITQ
                *** *         *           * *                 ** *   **  *   * * * *****  * **
            236 QMRALNPVIFINNLGDLQTITVGEDGITLGAGVTYSQAFKTIAEHFPPLARLFDRLGGEQVRNMGTIGGNIANGSPIGDT
            215 ALRDLPEVAFLSHCKDLAQIRETPDGYGIGAGVTIAALRAFAEGPHPALAGLLRRFASEQVRQVATIGGNIANGSPIGDG
            234 FHRELPVMIYVGHIREMKRIEVGANCLEIGAATPLTDCYQALAADYPDFGELLQRFASLQIRNQGTLGGNIGNASPIGDA
            227 RHQDIPLIIAIGQIEALRQVSWQGDRLVIGASAALSDLYPMLANYHPAFGELLARFASQQIRNQGTLGGNIANASPIGDG
                  * **  *       *       *   ***             *    ** **** * ** ** ****** *****
            316 PPALIALGATLTLRSSSGGRSLPLESYFIDYGKQDRLSGEFVEKLFIPFQKPESRYAVYKISKRRDEDISALCAAFNLTL
            295 PPALIAMGASLTLRRGQERRRMPLEDFFLEYRKQDRRPGEFVESVTLPKSAPGLR--CYKLSKRFDQDISAVCGCLNLTL
            314 PPLLIALGAKIVLRRGERRRELPLEEYFLDYKVTAREEGEFIEKILVPRARPSQAFKAYKVSKRIDDDISAVCAAISLDL
            307 APLLLALDALLILRCGATQRELPLNDFFLGYRQTALQPGEFIESIVLPASAP-ADFRAWKVSKRLEDDISAVCGAFNLTF
                ** ****** * ** *   * ****  ** *    *  *** *    *   *      ** *** * ****** * ****
            396 DAEGTVEDILIAFGGMAGTPKRATHLEAALLGKEWSQETIDAARDALDEDFTPLTDWRATAEYRQLTAKNLLTRFFLETS
            373 KGS-KIETARIAFGGMAGVPKRAAAFEAALIGQDFREDTIAAALPLLAQDFTPLSDMRASAAYRMNAAQAMALRYVRELS
            394 EDG-RIARARVAFGGMAAIPKRAAACEAALQGARLEAASFERAAAALANDFTPLSDFRASKEYRLLTAQNLLRKCFLELH
            386 DQG-VIRSARVAFGGMAATPKRAELCEQQLVGQPWRRETLEQAAQALAQDFTPLSDFRASSAYRLLVAQNLLRRYFIALT
                     *   * ******  ****   **** *          *   ** ******* ***  ** * *****  ** **
            476 GEKQELSRFSLEEA
            452 GEAVAVLEVMP
            473 APAVETRVTAYV
            465 APQLAIEVTAHE
```

Fig. 2. Alignment of the derived amino acid sequence of *Serratia proteamaculans* (*Sp*, accession) xanthine dehydrogenase (Xdh) subunits A and B with the enzymes of *Agrobacterium tumefaciens* (*At*, NP532983), *Rhodobacter capsulatus* (*Rc*, CAA04470) and *Pseudomonas aeruginosa* (*Pa*, NP250214). The completely conserved amino acids are denoted with an asterisk.

```
XdhB At 1 ----------------------------------------MTGSAEYIDDIPEPAGLVHGALGLADRAHAEIVSMDLSEVEA
XdhB Rc 1 ----------------------MSVGKPLPHDSARAHVTGQARYLDDLPCPANTLHLAFGLSTEASAAITGLDLEPVRE
XdhB Pa 1 MSNHRKPHKSQEELADLFRAELTTGVGRSVKHESAPKHVSGEAIYIDDRLEFPNQLHVYARLSERAHARITRLDVTPCYQ
XdhB Sp 1 MSNRKGAEHTQEHLAERFRQSLTSGVGRSRKHESADKHVSGEAQYIDDRLEFPNQLHLAARLSERAHAQIEKLDLSACYD
                                    **    * **  ** * * ****  *  * ** *  ** **** *  ***
       43 TPGVLWVMVGKDVPGENDVSSGGRHDEPLLAETKVEFHGQPIFAVFAETRDIARKAARKAKITYRDLPHFTDIDTAIENG
       58 SPGVIAVFTAADLPHDNDASP-APSPEPVLATGEVHFVGQPIFLVAATSHRAARIAARKARITYAPRPAILTLDQALAAD
       81 FPGVAIALTAADVPGQLDIGP-VVAGDPLLADGKVEYVGQMVLAVAADSLETARKAAMAAIVEYEDLEPVLDVVEALRKR
       81 FPGVVRVITWQDVPGELDIAP-LTHGDPLMAKDKVEYVGQVIAVVAAEDPEIAWRAAQAIKVTYRDLPARLDVTQSLREG
           ***  * *  ****  *  *      ****  *** *** *  ***     ** **  *  ** ***  *    **
      123 GALVIDPMTLKRGDAKIEMDVAPRRLTGTMRIGGQEHFYLESHIAMAVPGEDDEVTLWSSTQHPSEIQHIVSHILQVPSN
      137 SRFEGGPVIWARGDVETALAGAAHLAEGCFEIGGQEHFYLEGQAALALPAEGG-VVIHCSSQHPSEIQHKVAHALGLAFH
      160 -HFVLDSHQHRIGDSAAALAGAPHRLQGTLHIGGQEHFYLETQISSVMPSEDGGMIVYCSTQNPTEVQKLVAEVLGVSFN
      160 -FLVQEAHRHQRGDADRALAQAKHRIQGELHVGGQEHFYLETQIASVMPAEDGGMLVYSSTQNPTEIQKLVASVLNLPMH
             *        **   *** * **  *   ********** ***   * ***      *** * ***  **  *
      203 AVTVQVRRMGGGFGGKETQGNQFAALCAIAAKKLNRAVKIRPDRDEDMTATGKRHDFRVDYELGFDEEGRIHAVDATYAA
      216 DVRVEMRRMGGGFGGKESQGNHLAIACAVAARATGRPCKMRYDRDDDMVITGKRHDFRIRYRIGADASGKLLGADFVHLA
      239 RIVIDMRRMGGGFGGKETQAAAPACLCAVVAYHTGRPAKMRLPRMEDMQITGKRHPFYVEYDVGFDDDGRLHGIQIDLAG
      239 RVTIDTRRMGGGFGGKETQAAGPACLCAVMVYLTGRPVKMRLNRRDDMLITGKRHPFYIQYDVGFDDSGLLHGVKISLAG
           *    *************    * **** *  **** ***  *  ** ****** *   *  ***  * ***     *
      283 RCGFSSDLSGPVTDRALFHADSSYFYPHVHLTSRPLKTHTVSNTAFRGFGGPQGMLGAERFIEEIAYAVGKDPLDIRKLN
      296 RCGWSADLSLPVCDRAMLHADGSYFVPALRIESHRLRTNTQSNTAFRGFGGPQGALGMERAIEHLARGMGRDPAELRALN
      319 NCGYSPDLSGSIVDRAMFHSDNAYFLGNATINGHRCKTNTASNTAYRGFGGPQGMVAIEEIMDAVARSLGKDPLEVRKLN
      319 NCGYSLDLSGSIVDRAMFHADNAYFLEDVLITGHRCKTHTASNTAYRGFGGPQGMMAIEQVMDHIARYLALDPLAVRKTN
           ** * ****   ********  **     *  ** **** **** *********   *      **  * ***  ****
      363 FYGETGSG----------------RTTTPYHQEVEDNIIARVVEELETSSDYRARREAIIEFNRTSPIIRKGIALTPVKF
      376 FYDPPERGGLSAPPSPPEPIATKKTQTTHYGQEVADCVLGELVTRLQKSANFTTRRAEIAAWNSTNRTLARGIALSPVKF
      399 YYGKDE------------------RNVTHYHQTVEHNLLAEMTAELEASSEYARRREEIRAFNAASPVLKKGLALTPVKF
      399 YYGKDQ------------------RNVTHYHQPVEQNLLQEITAELEQSADYQARRQAIRQFNAQNPILKKGLALTPVKF
           **                     *  ***** ** * * *   *** *  *  **  *  **   * * ** *******
      427 GISFTMTAFNQAGALVHIYNDGSIHLNHGGTEMGQGLYTKVAQVVADAFQVDIGRVKITATTTGKVPNTSATAASSGTDL
      456 GISFTLTHLNQAGALVQIYTDGSVALNHGGTEMGQGLHAKMVQVAAAVLGIDPVQVRITATDTSKVPNTSATAASSGADM
      461 GISFTATFLNQAGALIHIYTDGSIHLNHGGTEMGQGLNTKVAQVVAEVFQVDVERIQITATNTDKVPNTSPTAASSGTDL
      461 GISFTAGFLNQAGALVLVYTDGSIQLNHGGTEMGQGLNTKVAQIVAEVFQVDIERIQITATDTGKVPNTSPTAASSGTDL
          ***** * ******** ******* ************ ******** *****     **** * ****** *********
      507 NGMAAYDAARQIRERLVKFAAENWNVPEEEVVFLPNRVRIGLEEIAFNDFIKKAYFARVQLSAAGFYKTPKIHWDRAAGR
      536 NGMAVKDACETLRGRLAGFVAAREGCAARDVIFDAGQVQASGKSWRFAEIVAAAYMARISLSATGFYATPKLSWDRLRGQ
      541 NGKAAQNAAETIKRRLVEFAARHWKVSEEDIEFRNNQVRIRELILPFEELIQQAYFGQVSLSSTGFYRTPKIFYDREQAR
      541 NGKAAENAALIIKQRLIEMLSKQHQVSAEQIIFNNGQVKVAERYFSFEQVVEQAYFNQVSLASTGYYRTPKIFYDRDQAR
          ** **  **  *  **  * *       *   *   **        *      ***  **** **** ****  **
      587 GTPFYYFAYGAACSEVSIDTLTGEYMMERTDILHDVGKSLNPAIDIGQIEGAFVQGMGWLTTEELWWDGKGRLRTHAPST
      616 GRPFLYFAYGAAITEVVIDRLTGENRILRTDILHDAGASLNPALDIGQIEGAYVQGAGWLTTEELVWDHCGRLMTHAPST
      621 GRPFYYFAYGAACSEVIVDTLTGEYRMLRTDILHDVGDSLNPAIDIGQVEGGFVQGMGWLTMEELVWNAKGKLMTSGPAS
      621 GHPFYYFAYGAACAEVVIDTLTGEYKLLRADILHDVGDSLNPAIDVGQVEGGFVQGMGWLTSEELVWDEQGKLLTNGPAS
          * ********** ** ********  ********** ********** ** ********* ******  * * *  *
      667 YKIPLASDRPKSFNVKLAEWAENAEPTIGRSKAVGEPPFMLAISVLEALSMAVASVADYKVCPRLDAPATPERVLMAVER
      696 YKIPAFSDRPRIFNVALWD-QPNREETIFRSKAVGEPPFLLGISAFLALHDACAACGPH--WPDLQAPATPEAVLAAVRG
      701 YKIPAVADMPLDLRVKLLENRKNPEQTVFHSKAVGEPPFMLGISVWCAIKDAVASLADYRAQPAIDAPATPERVLWGVEQ
      701 YKIPAIGDVPADLRVRLLENRKNPEDTVFHSKAVGEPPFMLGISVWCAIKDAVASLADYRLQPNIDAPATPERVLWGVQQ
          *****  * *    * * *   * * * * ***************  *  ***** ***   *  **********  *
      747 LKKV
      773 AEGRA
      781 MRRLKAAQAQAADAAVEPA
      781 MLHGDVVTDDVRGNGDGRVD
```

Fig. 2 (*continued*)

we hypothesized that the *S. proteamaculans* enzyme responsible for the C8-oxidation of adenine and the cytokinins was xanthine dehydrogenase (Xdh, EC 1.1.1.204). Previous work on *Rhodobacter capsulatus* Xdh indicated that the enzyme was encoded by two overlapping genes, *xdhAB* (Leimkühler et al., 1998). The *xdhA* gene encodes the subunit responsible for binding the iron–sulfur cluster and FAD while the XdhB subunit has the molybdopterin-binding site. Degenerate oligonucleotide primers (see Section 4) were designed based on conserved regions of amino acids in an alignment of the Xdh subunits of *Agrobacterium tumefaciens*, *Rhodobacter capsulatus* and *Pseudomonas aeruginosa*. A PCR product of ca. 750 bp was amplified from *S. proteamaculans* B1 genomic DNA using the primers designed for *xdhB*. The derived amino acid sequence from this PCR product had high similarity to the other bacterial XdhB subunits (data not shown). Radioactively labeled PCR product was used to screen a cosmid library of *S. proteamaculans* B1 DNA in *E. coli*. The *xdhAB* genes (DQ417495) were found on a 5.7 kb *Eco*R1 fragment of the isolated cosmid. The XdhA subunit was encoded in a 1431 bp open reading frame (ORF) while XdhB was encoded by a 2403 bp ORF, the two ORFs overlapped by seven bp. An alignment (Fig. 2) of the derived amino

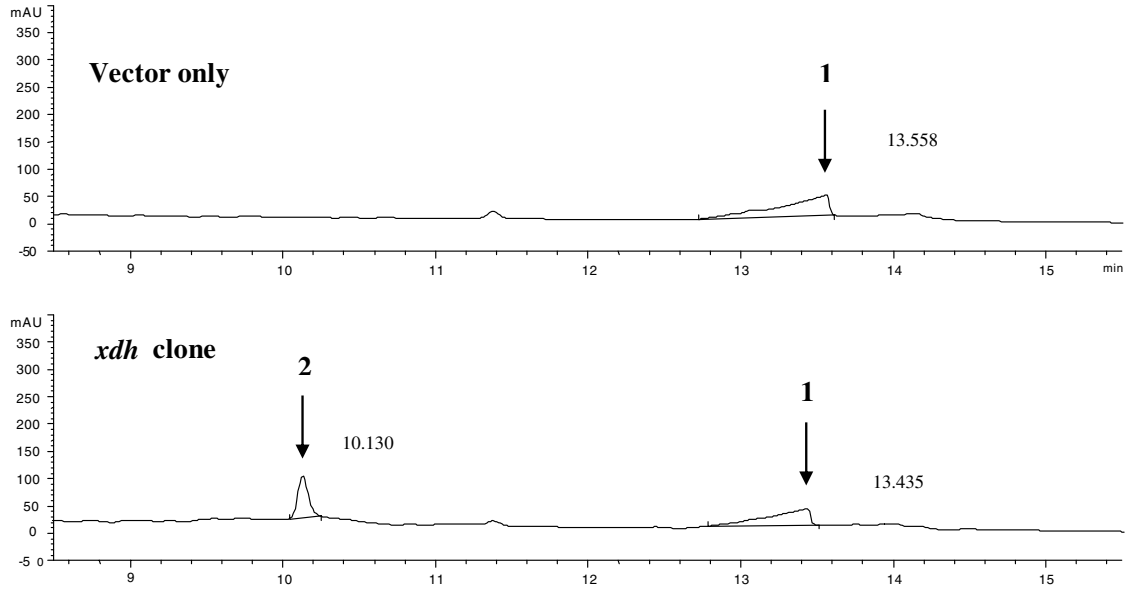

Fig. 3. Biotransformation of N^6-benzyladenine (**1**) to 8-hydroxy-N^6-benzyladenine (**2**) by an *Escherichia coli* isolate expressing the *Serratia proteamaculans* (*Sp*) xanthine dehydrogenase (*xdh*) genes A and B.

acid sequences from the *S. proteamaculans* and other bacterial *xdhAB* genes showed numerous stretches of highly conserved amino acids, particularly in the molypterin-binding subunit.

To confirm that the *S. proteamaculans* Xdh could catalyze the C8-oxidation of cytokinins the *E. coli* isolate carrying the cloned DNA and an isolate containing the cloning vector alone were incubated in M9 minimal media containing 0.25 mg/ml **1** for three days at 30 °C. The culture filtrates were fractionated by RP-HPLC and the results were compared. Only the filtrate from the isolate containing the *xdhAB* clone had a peak with the same retention time as **2** (Fig. 3). The UV scan of this peak corresponded exactly to that of **2** (data not shown).

3. Concluding remarks

To the best of our knowledge these are the first reported metabolites of cytokinins resulting from biotransformation by *S. proteamaculans*. Moreover, they are the first hydroxylated cytokinin metabolites reported to be natural products.

4. Experimental

4.1. General experimental procedures

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. All solvents were HPLC grade and were used as such. Additional N^6-benzyladenine was purchased from Caisson Laboratories, Inc., USA. Analytical HPLC analysis was carried out on a high performance liquid chromatograph 1100 series (Hewlett–Packard, Palo Alto, CA) equipped with a quaternary pump, automatic injector, diode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (4.6 i.d. × 250 mm, 5 μm particle size silica), equipped with an in-line filter. The RR_t is reported for a linear gradient elution with H_2O–CH_3CN, (90–0% H_2O) for 30 min at a flow rate of 1.0 ml min^{-1}.

Diaion HP-20 resin and reversed-phase octadecyl-functionalized silica gel (RP C-18) were used for column chromatography. The prep. TLC was carried out on RP C-18 (20 × 20 cm × 0.25 mm) plates. The TLC was performed on silica gel aluminum sheets (Kieselgel 60 F_{254}, 5 × 2 cm × 0.2 mm) and RP C-18 plates. Compounds were visualized under UV light and by dipping the plates in a 5% aqueous (wt v^{-1}) phosphomolybdic acid solution containing 1% (wt v^{-1}) ceric sulphate and 4% (v v^{-1}) H_2SO_4, followed by charring on a hot plate.

Electron impact (EI) mass spectra were obtained on a VG 70-VSE magnetic sector spectrometer operating with the electron energy of 70 eV and a source temperature of 200 °C, using a solids probe. High resolution mass measurements were performed either using EI or on a Q-ToF Global Ultima mass spectrometer (Waters/Micromass, Manchester, UK), fitted with a nano-lockspray option and nano-electrospray source. Data were acquired in positive ion mode and analyzed using MassLynx 4.0 spectrum program (Waters/Micromass).

Fourier transform IR spectra were obtained on a Perkin–Elmer Paragon 100 spectrometer using a diffuse reflectance cell.

NMR spectra were obtained on a Bruker Avance DRX 500 MHz spectrometer (Bruker Biospin Ltd., Milton, ON, Canada), equipped with a cryoprobe. For ^1H (500 MHz) δ

values were referenced to $(CD_3)_2SO$ ($CD_2HS(O)CD_3$, 2.50 ppm) and for ^{13}C (125.8 MHz) referenced to $(CD_3)_2SO$ (39.5 ppm). For ^1H NMR, spin coupling constants (J values) were reported to the nearest 0.5 Hz.

4.2. Soil sampling and enrichment

Individual soil samples were collected from fields in central Saskatchewan in July 2002. The crops growing in the fields included bean, lentil, chickpea, pea, barley, spring and winter wheat and canola. The soil was collected in an approximately 6 in. radius of individual plants. Eighty-five grams of soil from each of the eight crop fields was added to sterile deionized H_2O (100 ml) that contained either *Brassica napus* L. cake (10 g) or milled *Pisum sativa* L. (10 g). The *B. napus* (Echo) cake, containing approximately 12% oil, was prepared by M. Reaney (Agriculture and Agri-Food Canada, Saskatoon Research Centre) in an expeller mill. The milled *P. sativa* (CDC Mozart) was obtained from P. Polowick (NRC Plant Biotechnology Institute). The soil suspensions were incubated at room temperature with shaking at 170 rpm for two weeks. The suspensions were centrifuged for 15 min at 10,000*g* and the excess liquid was decanted. The samples were freeze-dried and stored at −80 °C.

4.3. Recovery of microorganisms and culture conditions

The soil samples from the eight crop fields were pooled by combining one gram each of the freeze-dried material. The pooled samples were suspended in sterile deionized H_2O (160 ml) in a Waring blender and dispersed on low speed (3 × 20 s). The solution was transferred to a 250 ml centrifuge bottle and underlaid with 40 ml Nycodenz (1.3 g ml^{-1}) [5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-*N*,*N*′-bis(2,3-dihydroxypropyl)isophthalamide]. The solution was centrifuged for 30 min at 10,000*g* at 4 °C. The microbial cells form a cloudy layer on the Nycodenz cushion (Bakken and Lindahl, 1995). The clear upper liquid is removed and the microbial layer is recovered. Various dilutions of the recovered solution were plated on tryptic soy broth (TSB) and incubated overnight at 30 °C to get an estimate of the cfu. For selection of microorganisms able to metabolize **1**, approximately 10,000 cfu each were plated onto six 150 mm Petri dishes of M9 minimal medium (Sambrook and Russell, 2001) containing 2 mg ml^{-1} **1** and 100 μg ml^{-1} glucose. The plates were incubated for one week at 30 °C. Colonies with distinct morphologies displaying vigorous growth on the selective medium were isolated by streaking onto the selective medium. A single isolate (B1) continued to show vigorous growth on the medium containing **1**. Small-scale (10 ml) liquid cultures of B1 were done in M9 minimal medium containing 1 mg ml^{-1} **1** and 100 μg ml^{-1} glucose. The cultures were incubated at 30 °C with shaking (150 rpm) and aliquots (1 ml) were removed at 48 h intervals for HPLC analysis.

4.4. Identification of microbial isolate B1 and cloning of *xdhAB*

Colony PCR was performed on isolate B1 to amplify the DNA encoding the 16S ribosomal RNA. The primers used were 16S-27F = 5′-AGAGTTTGATCMTGGCTCAG-3′ and 16S-1492R = 5′-TACGGYTACCTTGTTACGACTT-3′ (M = C or A; Y = C or T) (Suzuki and Giovannoni, 1996). The 20 μl reactions contained 200 μM each deoxynucleoside triphosphates, 5 pmole each primer, 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 0.1% (v v^{-1}) Triton X-100, and 0.5 U *Taq* polymerase (Invitrogen, USA). The amplifications were performed in a MJ Research thermal cycler using the following program: (1) 95 °C hold for 5 min, (2) 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min; and (3) a final extension at 72 °C for 5 min. The resulting PCR product was cloned into pCR2.1-TOPO (Invitrogen, USA) and sequenced. The sequencing reactions were performed using BigDye chemistry and analyzed on an ABI377 sequencing apparatus (Applied Biosystems, USA). BLAST analysis of ca. 500 bp of sequence each from both ends of the ca. 1500 PCR product showed greater than 99% similarity to 16S ribosomal RNA of *S. proteamaculans* (Altschul et al., 1990).

To design degenerate oligonucleotide primers to amplify the gene encoding the XdhB subunit the derived amino acid (aa) sequences of the genes from *Agrobacterium tumefaciens* (NP532983), *Rhodobacter capsulatus* (CAA04470) and *Pseudomonas aeruginosa* (NP250214) were aligned using the Clustal W algorithm (Thompson et al., 1994). Based on conserved regions in the alignment the following mixed primer sets were synthesized: xdhB-F2 = 5′-AAYCAYGGNGGNACNGARATG (corresponds to aa sequence NHGGTEM) and xdhB-R2 = 5′-AANGGNGGYTCNCCNACNGCYTT (corresponds to aa sequence KAVGEPPF), wherein Y = C or T, R = A or G and N = A, C, T, or G. PCR amplification of *S. proteamaculans* genomic DNA (20 ng) was carried out with each of the mixed primers (10 pmol) using the following program: (1) an initial hold at 94 °C for 3 min, (2) 40 cycles of 94 °C for 20 s, 63.5 °C for 30 s, and 72 °C for 1 min and (3) a final extension at 72 °C for 5 min. The reaction yielded a single PCR product of ~750 bp that was cloned into pCR2.1-TOPO. A cosmid library of *S. proteamaculans* genomic DNA was constructed in pWEB and transformed into *E. coli* EPI100 (Epicentre Technologies). A radioactively labeled *xdhB* PCR product was used in hybridizations to identify, isolate and subclone the *xdhAB* genes from the library. Sequencing was performed as described above.

4.5. Isolation of biotransformation products of benzyladenine (**1**), adenine (**4**) and isopentenyladenine (**5**)

For isolation of metabolites **2**, **3** and **6** from *S. proteamaculans* culture filtrates, the bacterium was cultured for two days in TSB at 30 °C with shaking (150 rpm). The cells

were collected by centrifugation, the TSB was decanted and the pellet was resuspended in M9 minimal medium. This cell suspension was inoculated into 1 l M9 minimal medium containing 0.25 mg ml^{-1} **1**, **4** or **5**. The cultures were incubated at 30 °C with shaking (150 rpm) for one week. The cells were removed by centrifugation and the medium was filtered through a 0.45-μm filter (Nalgene, USA). For **1**, the filtrate was freeze-dried and this material (1.2 g) was reconstituted in distilled H_2O (100 ml). For **4** and **5**, the filtrates were concentrated by evaporation (up to 100 ml). The filtrates were fractionated on a column packed with Diaion HP-20 resin. The column was eluted successively with H_2O (500 ml), H_2O–MeOH (1:1, 500 ml) and MeOH (500 ml). Portions (100 ml) were collected, concentrated to dryness and analysed by HPLC. Analysis of HPLC chromatograms showed a major metabolite (**2**, $R_t = 9.8$ min; **3**, $R_t = 7.3$ min; and **6**, $R_t = 12.6$ min), as well as some minor peaks. Combined fractions (230 mg for **2**, 312 mg for **3**, and 74 mg for **6**) that contained the metabolite of interest were further fractionated by RP C-18 silica gel CC using a gradient elution of H_2O–CH_3CN (100–50% H_2O). After HPLC analysis of the resulting fractions, the metabolite was purified by RP C-18 prep. TLC (H_2O–CH_3CN, 1:1). The purity of the isolated compound (42.8 mg of **2**, 10.2 mg of **3**, and 20 mg of **6**,) was confirmed by HPLC and ^1H NMR spectroscopic analyses.

4.6. Hydrogenolysis of 8-hydroxybenzyladenine (2)

Five percent Pd/C (1 mg) was added to a solution of 8-hydroxy-N^6-benzyladenine (**2**, 3 mg, 0.01 mmol) in MeOH (1 ml) at 50 °C and the resulting suspension was vigorously stirred under H_2 (balloon pressure). After 12 h, the mixture was filtered through Celite and the combined filtrate and washings were concentrated. The residue was fractionated by prep. TLC (H_2O–CH_3CN, 1:1) to give 8-hydroxyadenine (**3**) (1 mg), with the recovery of 1.2 mg of **2**.

4.6.1. Spectroscopic data

N^6-Benzyladenine (***1***): ^1H NMR (500 MHz, $(CD_3)_2SO$) δ 4.69 (*br s*, 2H), 7.19 (*dd*, $J = 7.0$ Hz, 1H), 7.28 (*dd*, $J = 7.5$ Hz, 2H), 7.33 (*d*, $J = 7.5$ Hz, 2H), 8.10 (*s*, 1H), 8.16 (s, 1H), 8.10–8.16 (*br s*, 1H, D_2O exchangeable), 12.85 (*br s*, 1H, D_2O exchangeable); ^{13}C NMR (125.8 MHz, $(CD_3)_2SO$) δ 43.3, 127.3, 127.6, 128.8 (2×), 139.7 (2×), 140.2, 152.9, 154.3; HREIMS *m/z* measured 225.1009 (225.1014 calcd. for $C_{12}H_{11}N_5$); MS-EI *m/z* (% relative intensity): 225 (M^+, 100), 120 (11), 106 (59); UV (λ_{max}, CH_3OH) 211 (log ε 4.41), 271 (log ε 4.29).

8-Hydroxy-N^6-benzyladenine (***2***): For ^1H NMR and ^{13}C NMR spectra, see Table 1; HREIMS *m/z* measured 241.0965 (241.0964 calcd. for $C_{12}H_{11}N_5O$); MS-EI *m/z* (% relative intensity): 241 (M^+, 100), 223 (7), 164 (9), 136 (11), 106 (68), 91 (84); FTIR ν_{max}: 3361, 3031, 1717, 1650, 1628, 1517, 1442, 1356, 612 cm^{-1}. UV (λ_{max}, CH_3OH) 213 (log ε 4.30), 276 (log ε 4.04).

8-Hydroxyadenine (***3***): ^1H NMR (500 MHz, $(CD_3)_2SO$) δ 11.25 (*s*, 1H), 9.89 (*s*, 1H), 7.95 (*s*, 1H), 6.33 (*s*, 2H); HR-MS *m/z* measured 152.0563 (152.0566 calcd. for $C_5H_5N_5O$).

8-Hydroxy-N^6-isopentenyladenine (***6***): For ^1H NMR and ^{13}C NMR see Table 2; HREIMS *m/z* measured 219.1122 (219.1120 calcd. for $C_{10}H_{13}N_5O$).

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