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Unusual production of glutathione in *Actinobacteria*

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Abstract

Most *Actinobacteria* produce mycothiol as the major thiol. In addition to mycothiol *Rhodococcus* AD45 generates a substantial level of glutathione possibly using genes acquired in a lateral transfer. Instead of mycothiol, *Rubrobacter radiotolerans* and *Rubrobacter xylanophilus* produce glutathione, whose synthesis appears to involve enzymes substantially different from those in other organisms.

Keywords

mycothiol; actinomycetes; glutathione; *Rubrobacter*; *Rhodococcus*

Low molecular weight thiols, like glutathione, are critical for aerobic cells, since they maintain the required redox balance for normal cellular processes and protect against various toxins and oxidative stress (Ghezzi, 2005; Masip *et al*., 2006). While most eukaryotes and gram-negative bacteria contain glutathione as the dominant thiol, prior surveys of *Actinobacteria* have demonstrated that mycothiol, not glutathione, is the main thiol in an overwhelming majority of these organisms (Newton *et al*., 1996). Previously, the only actinomycetes reported to contain glutathione were *Nocardiopsis flava* ATCC 29533 (Newton *et al*., 1996) and *Rhodococcus* AD45 (van Hylckama Vlieg *et al*., 1998). Also, there have been reports of glutathione dependent enzymes (Grund *et al*., 1990; van Hylckama Vileg *et al*., 1999, Dhar *et al*., 2003) in certain strains of *Actinobacteria*. Herein, we examine the thiol composition of selected *Actinobacteria* and report some unexpected findings.

Cultures were grown on Tryptic Soy (TS) broth, since this medium contains only traces of glutathione (Newton *et al*., 1996), shaken at 220 rpm until the cells reached mid-log phase growth when they were harvested by centrifugation for 10 min at 4,500 × g at 4°C. The exception was *Kineococcus radiotolerans,* which was grown on TS agar plates, removed by scraping and pelleted for thiol analysis. All strains were incubated at 30°C, except *Mycobacterium smegmatis* and *Rubrobacter radiotolerans,* which were incubated at 37°C, and *Rubrobacter xylanophilus,* which was incubated at 55 °C. Before harvesting, the purity of the cultures was checked by examining colony morphology and gram stain. Thiol derivatization with monobromobimane (mBBr) and analytical high-performance liquid chromatography were performed according to protocols previously described for mycothiol determination (Rawat *et al.*, 2003) and CoA analysis (Fahey & Newton, 1987). Purified derivatized mycothiol and glutathione derivatized with mBBr served as standards.

As a reference, we redetermined the thiol content of *M. smegmatis* and obtained values consistent with those reported earlier (Table 1). We also reanalyzed the thiol content of

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Nocardiopsis flava ATCC 29533 and were unable to detect any glutathione although the level of mycothiol was in the same order of magnitude (1.3 ± 0.3 μmol/g dry weight versus 2.7 μmol/g dry weight as previously reported) (Table 1). The discrepancy in the glutathione content between the two studies may have been due to contamination of the *N. flava* culture in the previous study (Newton *et al*., 1996). Other mycothiol dominant species are included in Table 1 and a careful examination failed to detect glutathione. These include *Streptomyces griseus* ATCC 13273, from which two glutathione *S*-transferase isoenzymes with activity against the standard glutathione *S*-transferase substrate, 1-chloro-2,4-dinitrobenzene (CDNB), have been purified (Dhar *et al*., 2003), and *Streptomyces ghanensis*, which requires glutathione for growth on *m*-hydroxybenzoate, presumably for the isomerization of maleylpyruvate (Grund *et al.*, 1990). In both these strains, mycothiol content and enzymatic activity using mycothiol as a substrate were not measured; since glutathione is absent in this genus, the *in vivo* thiol substrate for these enzymatic reactions is unlikely to be glutathione.

Rhodococcus sp. AD45 is a strain that can use isoprene as the sole source of carbon and energy and can oxidize many epoxides and chlorinated ethenes to chlorinated epoxyethanes (van Hylckama Vlieg *et al*., 1998; van Hylckama Vlieg *et al*., 1999). A glutathione *S*-transferase, IsoI, involved in the metabolism of these compounds has been purified (van Hylckama Vlieg *et al*., 1999). We find that *Rhodococcus* sp. AD45 produces a very high level of mycothiol but also a significant level of glutathione throughout its growth cycle (Table 1). The level of glutathione (2.0 μmol per g residual dry weight) is comparable to that reported by Van Hycklama Vlieg *et al*. (1998) based upon the determination of the adduct formed with 1,2-epoxyhexane. In contrast to strain AD45, only mycothiol and no glutathione was detected in *Rhodococcus* sp. Rha1 (Table 1) or *Rhodococcus erythropolis* (Table 1). A gene sequence encoding the two glutathione biosynthesis enzymes, two glutathione *S*-transferases, and other enzymes required for isoprene metabolism has been identified in *Rhodococcus* AD45 by van Hylckama Vlieg *et al.* (2000). This suggests that in conjunction with a possible horizontal gene transfer of isoprene degradation genes, there has been a transfer of the glutathione biosynthetic pathway to support the glutathione *S*-transferase activity required for this pathway.

Given the high concentration of mycothiol found in *Rhodococcus* sp. AD45 it is surprising that no mycothiol adduct was seen in the earlier study (van Hylckama Vlieg *et al*., 1998). Mycothiol *S*-conjugate amidase cleaves an amide bond in mycothiol toxin adducts to produce mercapturic acids and 1*O*-(2-amino-2-deoxy-±-D-glucopyranosyl)-D-*myo*-inositol, which is recycled back to mycothiol. We determined the mycothiol amidase activity by reacting 100 μg of cell extract protein in 25 mM HEPES (pH 7.5), 3 mM β-mercaptoethanol with 30 μM of mycothiol-monobromobimane adduct (MS-MB) in a 50 μl reaction volume. The samples were incubated for 30 min at 30°C, and then the reaction was stopped by adding 50 μl of 40 mM methanesulfonic acid and the samples were subjected to HPLC analysis for detection of the bimane derivative of *N*-acetylcysteine. The mycothiol *S*-conjugate amidase activity (2.5 ± 0.1 nmol/min/mg protein) in *Rhodococcus* AD45 cell extracts was twice that of *M. smegmatis* (Newton *et al*., 2000). Thus, any mycothiol adducts in *Rhodococcus* AD45 would have been further metabolized to produce mercapturic acids (Newton *et al*., 2000). Alternatively, the thiol composition may have favored glutathione in that study where cells were grown on isoprene.

The most surprising finding of the present study is that *Rubrobacter radiotolerans* (Yoshinaka *et al*., 1973; Suzuki *et al*., 1998) and *Rubrobacter xylanophilus* (Carreto *et al*., 1996), both strongly radiation resistant (Ferreira *et al*., 1999), contain only glutathione (Table 1). This result was confirmed by analyzing thiol content with two different high-performance liquid chromatography protocols. Glutathione is not uniquely associated with radiation resistance since the radiation resistant *Kineococcus radiotolerans*, an actinomycete discovered at the Savannah River nuclear waste site (Phillips *et al*., 2002), contains mycothiol but no glutathione (Table 1). Interestingly, in the genus *Arthrobacter* in which *R. radiotolerans* was originally

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classified (Suzuki *et al*., 1998), there are species that do not contain either mycothiol or glutathione such as *A. globiformis* (Newton *et al*., 1996) and species such as *A. histodinolovorans* and *A. aurescens* that contain mycothiol (Table 1).

To identify the glutathione biosynthesis genes, the *R. xylanophilus* genome sequence (GenBank CP000386; Comprehensive Microbial Resource, www.cmr.org) was Blastp (Altschul *et al*., 1997) searched using representatives of the four major classes (Copley & Dhillon, 2002; Janowiak & Griffith, 2005) of glutathione synthetase (GshB). The GshB sequences from *Homo sapiens*, *Escherichia coli*, and *Nostoc* sp. produced no significant hits (E-value < 0.1). However, the C-terminal 390 amino acid sequence of the bifunctional *Streptococcus agalactiae* GshF protein (Janowiak & Griffith, 2005), that encodes the GshB equivalent, produced one weak hit, a 425 amino acid phosphoribosylamine:glycine ligase (Rxyl_0991; YP_643769), with E-value of $6e^{-4}$ that exhibited 29% identity in a 101 amino acid overlap. None of the *R. xylanophilus* annotated genes, including Rxyl_0991, contained the signature for prokaryotic GshBs, PFAM02951 (http://pfam.sanger.ac.uk/), although Rxyl_0991 and fifteen other proteins did possess the glutathione synthetase ATP binding domain, PFAM02955.

A Blastp search (Altschul *et al*., 1997) for possible γ-glutamylcysteine synthetase (GshA) sequences using the sequences from *E. coli*, *Homo sapiens*, *Xylella fastidiosa*, or the *N*-terminal 520 residues of the GshF protein sequence from *S. agalactiae* (May & Leaver, 1994; Janowiak & Griffith, 2005) produced no hits with E-values below 0.1. A search using the *E. coli* YbdK protein, which does not provide the principal GshA activity in *E. coli* but has been shown to have γ-glutamylcysteine synthetase activity (Lehmann *et al*., 2004), produced three hits, having E-values ranging from $3.8e^{-31}$ to $3.2e^{-41}$ and exhibiting 27-29% identity (44-49% similarity) over 343-360 residue overlaps with the 372 residues of the YbdK protein. A ClustalW alignment of the YbdK and the three *Rubrobacter* genes is shown in Fig. 1. These three proteins, Rxyl_0030 (YP_642823), Rxyl_1350 (YP_644125) and Rxyl_1127 (YP_643905), also contain the GCS2 domain (PF04107) that is present in all GshAs. However, members of the GCS2 superfamily are present in many strains, such as *M. smegmatis,* which lack glutathione and thus these genes may simply code for a carboxylate amine ligase of unknown function. Moreover, Blastp analysis with Rxyl_0030, Rxyl_1350 and Rxyl_1127 indicated that these genes are most similar to genes in *Gloeobacter violaceus*, an unicellular cyanobacterium with high GC content (62%), and the mycothiol containing GC rich actinomycetes, such as *Mycobacterium* species, which likely do not contain glutathione. Conversely, a gene, Rxyl_0031, annotated as a redoxin, with a thioredoxin superfamily domain (http://supfam.mrc-lmb.cam.ac.uk/), which encompasses glutathione S-transferases, glutathione peroxidase, and thiol tranferase domains, is present upstream of Rxyl_0030 and forms a bicistronic operon with it. Similarly, a gene annotated as a redoxin is present upstream but on the complementary strand of Rxyl_1127. Future experiments consisting of gene inactivation or recombinant protein expression are clearly needed to ascribe a role in glutathione biosynthesis for Rxyl_0030, Rxyl_1127, or Rxyl_1350.

Intriguingly, the *R. xylanophilus* genome contains two genes, Rxyl_0300 (YP_643088) and Rxyl_0239(YP_643028), which have both the N-terminal signature (PF02798) and a C-terminal signature (PF00043) required for glutathione *S*-transferase activity. Blastp analysis of Rxyl_0300 indicated that this gene is similar to many genes annotated as putative glutathione *S*-transferases. The top four hits with 61-70% sequence identity (77-79% similarity) and E-values ranging from $1.3e^{-75}$ to $5.9e^{-80}$ were from *Burkholderia* and *Ralstonia.* Blastp analysis of Rxyl_0239 also produced hits in genes annotated as glutathione S-transferases in the genera, *Burkholderia*, *Bordetella* and *Pseudomonas. Burkholderia*, *Ralstonia* and *Pseudomonas* strains are omnipresent and metabolically diverse and thus contribute to such processes as biodegradation of pollutants and biocontrol of root diseases; some, along with *Bordetella*

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species, also cause disease in plants, animals and humans. The need for glutathione detoxification activity may have provided the selective pressure for evolution of glutathione metabolism in *Rubrobacter.*

Rubrobacter branched early from the main line of descent leading to *Actinobacteria* (Kunisawa, 2007; Gao & Gupta, 2005), possibly before the evolution of mycothiol biosynthesis; it may have independently evolved the ability to synthesize glutathione or acquired the ability via a lateral gene transfer. Identification of the glutathione biosynthesis genes should clarify this issue and shed light on the evolution of glutathione in these organisms. The acquisition of genes involved in glutathione biosynthesis along with the complete isoprene degradation pathway in *Rhodococcus* AD45 suggests that lateral transfer of genes involved in glutathione biosynthesis may be important in polluted environments where bacteria need glutathione requiring degradation pathways for survival.

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Rxyl_0030    1 AE-AVSYELFQSVLEIKTPVCRTVGEAERVLRELRGRVGSWTAACGASLASAGTHPFSRY
Rxyl_1350    1 PEGTLASELSASCLEVRTPAYASVAELARALPALRARVRRLAERSGARLVSAGAHPFSPA
Rxyl_1127    1 FS----GELIDCEVEANTGVHREAAGVARDLLARRRTLLEHAGRLGRVLGTSGTHPLGDW
ybdK         1 TAGEVKHDITESMLELATDVCRDINQAAGQFSAMQKVVLQAATDHHLEICGGGTHPFQKW

Rxyl_0030   60 RDQKVTEHERYRQVIEELRWVATREVIFGQHVHVAVPGPEEAIQAHNRLAEQAPLLLALS
Rxyl_1350   61 AEQPITGKPRYRKVDEEMGWPARMQAIYGLHVHVAVPGGEEAVRAVSALARHVPLFIALS
Rxyl_1127   57 REQEIIDKPHYQYLKRKLGWLIRRNNTFSLHVHYAVQGKEKVIYLFDRIREYVPHFLAVS
ybdK        61 QRQEVCDNERYQRTLENFGYLIQQATVFGQHVHVGCASGDDAIYLLHGLSRFVPHFIALS

Rxyl_0030  120 ANSPYWQGMDTGFESSRVQIFE-TFPRAGMPPAFPEYAAFEAYVDLMVECGAMDDYTFCW
Rxyl_1350  121 ANSPFWEGRDTRLASVRAKVFG-LIPRSGLPPRFASWEEFVRHVERLVRAGSIRDYTFCW
Rxyl_1127  117 VNSPFWQGEFTDTRSARALVFSRSLPHAGMPEAFGSWSAYAGYLDFVGRPGVIRRLGEIW
ybdK       121 AASPYMQGTDTRFASSRPNIFS-AFPDNGPMPWVSNWQQFEALFRCLSYTTMIDSIKDLH

Rxyl_0030  179 WDVRPHPKLGTIELRVLDSQTHLRHAVALT--ALTQCIVASSLEDEDAPKGPYHRDIALE
Rxyl_1350  180 WDVRPHPKLGTVELRAPDAQTDPGRTAALA--ALCQCLAAAAEEFE-----PEDPLLTEE
Rxyl_1127  177 WDIRPHPRLSTLEIRAFDAQTDPARSEALISLAAATCDMLCAEYESGELRPARPVREIED
ybdK       180 WDIRPSPHFGTVEVRVMDTPLTLSHAVNMAG--LIQATAHWLLTERPFKHQEKDYLLYKF

Rxyl_0030  237 NKWRASRRGLDAAFFDVDERRNVPARDLARAAVERLRPHAQQLGCEEELLGVLEIVEGGS
Rxyl_1350  233 NKWRATRHGLEAELYDFSGQRTVAARRAAEELVGRLLPVARELGCEAELEGVLEISRSAT
Rxyl_1127  237 NKWSAQRHGLDGLFVDHETHEPVPARWAVERLAELAASSSRRD-----LSSLERLLEEPT
ybdK       238 NRFQACRYGLEGVITDPHTGDRRPLTEDTLRLLEKIAPSAHKIGASSAIEALHRQVVSGL

Rxyl_0030  297 -GSRRQREIYEKSGDFLDVVAFLIEGTRPALAGEPS
Rxyl_1350  293 -GADRQRAVLAREGSLKSVVDYLAEATA--------
Rxyl_1127  292 -ESERQLLVWRETGSVKEVARDIARRTRAAIPAT--
ybdK       298 NEAQLMRDFVADGGSLIGLVKKHCEIWAGD------
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Fig. 1.

ClustalW alignment of YbdK from *Escherichia coli* W3110 (BAA35221); Rxyl_0030 (YP_642823), Rxyl_1350 (YP_644125) and Rxyl_1127 (YP_643905) from *Rubrobacter xylanophilus.*

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Table 1

Thiol content of Actinomycetes.

Strain	Thiol content (μmol/ g residual dry weight) Mycothiol	Glutathione
Mycobacterium smegmatis mc^{2}155[*]	11.3 ± 0.2 (4)	<0.01
Streptomyces ghanensis ATCC 14672 [1]	1.4 ± 0.1 (28)	<0.01
Streptomyces griseus ATCC 13273 [1]	3.0 ± 0.4 (28)	<0.01
Nocardiopsis flava ATCC 29533	1.3 ± 0.3 (4)	<0.01
Rhodococcus erythropolis ATCC 4277	3.9 ± 0.3 (4)	<0.01
Rhodococcus RHA1 [2*]	3.0 - 9.0 (4)	<0.01
Rhodococcus AD45 [2*]	15 – 20 (4)	1 - 3 (4)
Arthrobacter histodinolovorans[*]	0.3 ± 0.0 (3)	<0.01
Arthrobacter aurescens[*]	1.0 ± 0.2 (3)	<0.01
Rubrobacter radiotolerans ATCC 51242	<0.01	5.3 ± 0.6 (3)
Rubrobacter xylanophilus DSM 9941[*]	<0.01	2.4 ± 0.2 (4)
Kineococcus radiotolerans ATCC BAA149	4.0 ± 0.4 (3)	<0.01
Escherichia coli DH5	<0.01	7. 7 ± 1.1 (3)

Samples were harvested during log phase growth. Values are means ± SEM (n).

[1]Mean of quadruplicate samples harvested during 7 days of growth

[2]Range of MSH levels during log phase of growth

[*]*Rhodococcus* Rha1 (a gift from by Dr. Julian Davies), *Rhodococcus* AD45 (a gift from Dr. D. Jansen), *Rubrobacter xylanophilus* DSM 9941(a gift from Dr. J. Batista), *Mycobacterium smegmatis* mc^{2}155 (a gift from Dr. W. R. Jacob, Jr.), *Arthrobacter histodinolovorans* and *Arthrobacter aurescens* (gifts from Dr. A. Wright).