VOL. 56 NO. 2, FEB. 2003 THE JOURNAL OF ANTIBIOTICS pp. 135 — 142

Functional Annotation of Putative Aminoglycoside Antibiotic Modifying Proteins in *Mycobacterium tuberculosis H37Rv*

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(Received for publication July 12, 2002)

The growing availability of sequences of bacterial genomes has revealed a number of open reading frames predicted by sequence alignment to encode antibiotic resistance proteins. The presence of these putative resistance genes within bacterial genomes raises important questions regarding potential reservoirs of resistance elements and their evolution. Here we examine four gene products encoding predicted aminoglycoside-aminocyclitol antibiotic modifying enzymes, two phosphotransferases and two acetyltransferases, derived from analysis of the genome sequence of *Mycobacterium tuberculosis* strain H37Rv with the goal of assigning biochemical function by purification of each protein and characterization of their ability to modify aminoglycoside antibiotics. Only one of these enzymes, the previously characterized aminoglycoside acetyltransferase AAC(2′)-Ic, displayed compelling aminoglycoside modifying activity. While the putative phosphotransferase encoded by the *Rv3225c* gene did display low levels of aminoglycoside kinase activity, the predicted kinase encoded by the *Rv3817* gene lacked any such activity. A potential aminoglycoside 6′-acetyltransferase, encoded by the *Rv1347c* gene, did not show antibiotic acylation activity but did demonstrate selective thioesterase activity with numerous acyl-CoAs. This activity, together with the genomic environment of the *Rv1347c* gene in a likely polyketide synthesis cluster, suggests a role for this protein in secondary metabolism and not in antibiotic modification. It was thus shown that only one of four putative aminoglycosides modifying enzymes derived from the whole genome sequencing of *M. tuberculosis* H37Rv showed sufficient predicted enzyme activity to be annotated as an aminoglycoside resistance element. This study demonstrates the necessity of biochemical annotation methods as a follow up to *in silico* sequence alignment-based methods of assigning gene product function.

The sequencing of numerous microbial genomes in recent years has opened up many new avenues of research, with contributions from various scientific disciplines and the application of various new technologies to aid in genome analysis (reviewed in[1,2]). An unexpected outcome of the availability of this sequence data is the prevalence of predicted antibiotic resistance genes within the genomes of many bacteria and archae as annotated by sequence similarity. While chromosomally encoded resistance elements have been previously associated with certain bacterial species, *e.g.* in the *Acinetobacter* genus, the preponderance of antibiotic resistance genes have been linked to mobile genetics elements such as plasmids, transposons and integrons. The number of genetic elements emerging from whole genome sequence analysis is therefore of significance to our understanding of the origins of antibiotic resistance and its ecology.

A case in point is the genomic data from various mycobacterial species. These organisms include the causative agents of some of the most deadly infectious diseases and antibiotic treatment is complex and increasingly difficult as multidrug resistant strains of, for example, *Mycobacterium tuberculosis* emerge. The genomes of three mycobacteria have been sequenced: *M. tuberculosis* H37Rv, a laboratory stain of the organism that causes tuberculosis, *M. tuberculosis* CDC1551 (http://www.tigr.org/), a multi-

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Table 1. Proteins homologous to the putative aminoglycoside modifying enzymes described in this study.

M. tuberculosis H37Rv Protein	Homologous Proteins [a]	% Identity [b]
Putative phosphotransferase Rv3817	Putative aminoglycoside phosphotransferase from *M. tuberculosis* CDC1551 (Mt3925.1)	100
	Putative aminoglycoside phosphotransferase from *M. leprae* (ML0086)	34[c] (197 a.a overlap)
	Aminoglycoside 3'-phosphotransferase Type V from *Streptomyces fradiae* (P00555)	23.9
	Aminoglycoside 3'-phosphotransferase Type V from Micromonospora chalcea (A53239)	23.5
	Aminoglycoside 3'-phosphotransferase Type II from *Pseudomonas aeruginosa* (S61209)	24.7
Putative phosphotransferase Rv3225c	Putative aminoglycoside phosphotransferase from *M. tuberculosis* CDC1551 (Mt3323)	97
	Putative aminoglycoside phosphotransferase from *M. leprae* (ML1376)	51[c] (141 a.a overlap)
	Aminoglycoside 3'-phosphotransferase Type V From *Escherichia coli* (P14509)	19.9
Putative acetyltransferase Rv1347c	Putative acetyltransferase from *M. tuberculosis* CDC1551 (Mt1389)	100
	Aminoglycoside *N*-6'-acetyltransferase Type II from *Pseudomonas aeruginosa* (A37201)	28 (143 a.a overlap)
	Aminoglycoside *N*-6'-acetyltransferase Type I from *Serratia sp.* (JC1322)	28 (145 a.a. overlap)
	Malonyl-CoA decarboxylase from *Saccharopolyspora erythraea* (A36965)	37 (158 a.a overlap)

[a] The proteins listed represent a subset of homologues identified for each of the putative modifying enzymes based on BLASTP sequence alignments[33]. Hypothetical and/or putative proteins have been omitted for clarity. Accession numbers for each protein are included in brackets.
[b] Percentage identity between the Rv3817 and Rv3225c proteins and their full length homologues were determined using the Clustal method[34] to calculate sequence pair distances with a PAM250 residue weight table. Percentage identity between Rv1347c and its homologues are based on BLASTP sequence alignments for the specified a.a. overlap length using default parameters.
[c] Percentage identities between Rv3817and Rv3225c proteins and the *M. leprae* pseudogenes indicated are based on BLASTX sequence alignments for the specified a.a. overlap length using default parameters.

drug resistant strain, and *Mycobacterium leprae*[4], the causative agent of leprosy. These bacteria show several potential antibiotic resistance genes within their genomes including aminoglycoside resistance elements (Table 1).

Resistance to the aminoglycoside class of antibiotics is prevalent in both Gram-positive and Gram-negative bacteria and is most commonly caused by enzymes that modify the drugs by either *O*-phosphorylation or *N*-acetylation (for review see reference 5). In mycobacteria, aminoglycoside acetyltransferase activity has been detected in isolates of *M. chelonei*, *M. kansasii*, *M. phlei*, *M. smegmatis* and *M. vaccae*[6~10] and phosphotransferase activity has been detected in *M. fortuitum*[9], although in many cases enzymatic activity did not correlate with observed resistance. The aminoglycoside 2'-*N*-acetyltransferase gene, *aac*(2')-*Ic* from *M. tuberculosis H37Rv*

has been cloned by AINSA *et al.*[11] in a study that showed the universal presence of *aac*(2′) genes of this type in mycobacteria. The AAC(2′)-Ic protein was subsequently characterized and shown to have aminoglycoside acetylating activity *in vitro*[12].

Analysis of the complete genome sequence of *M. tuberculosis* H37Rv[13] identified several genes encoding putative aminoglycoside modifying enzymes in addition to the known *aac*(2′)-*Ic*, including two putative phosphotransferases *Rv3817*, and *Rv3225c*, and one possible acetyltransferase *Rv1347c*. The *Rv3817* gene product shows homology to several aminoglycoside phosphotransferases found in a variety of bacteria, including type V APH(3′) enzymes from neomycin-producing *Streptomyces fradiae* and *Micromonospora chalcea*[14,15] as well as a type II APH(3′) protein from *Pseudomonas aeruginosa*[16]. Similarly, the second larger putative APH protein, Rv3225c, is homologous to a type I 3′-phosphotransferase from *Escherichia coli*[17]. The third and final putative resistance protein, a potential aminoglycoside acetyltransferase, Rv1347c, shares homology with a *Pseudomonas aeruginosa* type II aminoglycoside *N*-6′-acetyltransferase[18] as well as with a Type I AAC from *Serratia* sp.[19] and to the *C*-terminal portion of a bacterial malonyl-CoA decarboxylase[20].

The claim of aminoglycoside resistance suggested by *in silico* analysis of gene sequences requires biochemical evidence for validation, *i.e.* functional annotation. This study describes the characterization of putative aminoglycoside modifying enzymes from *M. tuberculosis* by overexpression in *E. coli*, purification, and enzymatic assay for aminoglycoside modification activity. We demonstrate that only the known aminoglycoside acetyltransferase AAC(2′)-Ic is a bona fide antibiotic resistance element.

Materials and Methods

General Materials

Assay reagents and antibiotics were from Sigma (St. Louis, MO, USA), with the exception of kanamycin and gentamicin, which were purchased from Bioshop (Burlington, ON, Canada). Dalfopristin was the gift of Aventis Pharmaceuticals. Oligonucleotide primers were synthesized at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Plasmid DNA encoding *aac*(2′)-*Ic* in a pET-23a backbone was the kind gift of Dr. JOHN BLANCHARD, Albert Einstein College of Medicine, Bronx, NY, USA.

Subcloning of Putative Aminoglycoside Modifying Enzymes

Cosmid DNA containing the genes for the putative aminoglycoside modifying enzymes Rv3225c, Rv3817c, and Rv1347c was provided by Dr. S. COLE at the Institut Pasteur. The two putative phosphotransferase genes were amplified from purified cosmid DNA using the following oligonucleotides: for *Rv3225c*, 5′-CGGAATTCATATGCGCTTTG CGAAGCTGTCCG-3′ and 5′-CGGAATTCAAGCTTCTAACATCCGGGC CTAGCGCC-3′ and for *Rv3817*, 5′-CGGAATTCATATGTCCTTCCCCTCATCGC-3′ and 5′-CGGAATTCAAGCTTAGCGTGAGCTGTCGTCTTC-3′. The putative acetyltransferase gene *Rv1347c* was amplified from cosmid DNA using oligonucleotides: 5′-CGGAATTCATATGACCAAACCCACATCCGCTGGC-3′ and 5′-CGGAATTCAAGCTTACGCAGCCGTGGTC GGAGC-3′. The genes were amplified with Vent DNA polymerase (New England Biolabs) and PCR conditions were 1 minute at 94°C, 1 minute at 58°C for *Rv1347c* and *Rv3225c* amplification or 56°C for *Rv3817* amplification, and 1.5 minutes at 72°C for 30 cycles. PCR reactions also contained 5% DMSO. Amplified DNA was then isolated and used for cloning into suitable overexpression vectors.

Plasmids pET15Rv3817 and pET15Rv3225c were constructed by ligation of the 753 bp and 1422 bp putative aminoglycoside phosphotransferase genes respectively with a pET15b vector (Novagen) using Nde I and Hind III restriction sites. For overexpression of non-His tagged proteins, plasmids pET22Rv3817 and pETRv3225c were constructed in a similar manner using the pET22b(+) backbone (Novagen). For protein overexpression of the putative acetyltransferase, plasmids pET15Rv1347c and pET22Rv1347c were similarly constructed by ligation of the 630 bp gene into either pET15b or pET22b(+) vectors, respectively, using Nde I/Hind III restriction sites. All constructs were transformed into $CaCl_2$ competent *E. coli* BL21(DE3) for subsequent overexpression and purification.

Protein Overexpression and Purification

The proteins were overexpressed by growing *E. coli* BL21(DE3) cells containing the appropriate constructs in 1 liter Luria Broth supplemented with 100 μg/ml ampicillin. Cultures containing the putative phosphotransferase genes were grown at 30°C or 37°C to an OD_{600}~0.6, whereupon enzyme overexpression was induced by the addition of 1 mM IPTG. Harvested cells were washed with 0.85% NaCl before storage at −20°C.

Purification of the hexahistidine tagged putative phosphotransferase proteins followed the procedure that we have previously used to purify HisAAC(6′)-APH(2″) using

Ni NTA agarose (Qiagen)[21] before pure protein was dialyzed against 50 mM Tris-HCl pH 8.0, 1 mM EDTA. Purification of the hexahistidine tagged Rv1347c protein followed the same general procedure, using 50 mM HEPES pH 7.5, 500 mM NaCl and 20 mM imidazole, 1 mM EDTA, 0.1 mM DTT, and 1 mM phenylmethanesulfonyl fluoride as lysis buffer, 50 mM HEPES pH 7.5, 500 mM NaCl and 20 mM imidazole as equilibration buffer, and a 0% to 50% linear gradient of 50 mM HEPES pH 7.5, 500 mM NaCl, and 250 mM imidazole for elution of HisRv1347c. Pure protein was pooled and dialyzed against 25 mM HEPES pH 7.5 and 2 mM EDTA.

AAC(2′)-Ic was purified as previously described[12].

Aminoglycoside Phosphotransferase Assays

Aminoglycoside kinase activity was assessed by using the PK/LDH assay described previously[23], with reactions scaled down to 250 μl to monitor any activity in 96 well microtitre plate format using a SpectraMAX PLUS spectrophotometer (Molecular Devices). The observed ATPase activity of HisRv3817 allowed us to determine Michaelis-Menten kinetics by a fit of collected data by non-linear least fit squares to Eq. 1, using Grafit version 4.0[24]:

$$v=(k_{cat}/\mathrm{Et})[\mathrm{S}]/(K_m+[\mathrm{S}]) \quad (1)$$

The potential for low level aminoglycoside kinase activity was assessed by using the ^{32}P ATP phosphocellulose binding assay described previously[23].

Protein Kinase and Autophosphorylation Assays

We also tested the enzymes for the potential to phosphorylate Kemptide, Crebtide, or MARKS peptides, as well proteins histone H1 or MBP following the same general procedure described previously[23].

Aminoglycoside Acetyltransferase Assays

To assess whether Rv1347c could modify aminoglycosides, we monitored any observable acetyltransferase activity by the *in situ* titration of CoA with DTDP as previously described[22]. Assays performed monitored any modification of neamine, kanamycin A, bekanamycin, tobramycin, amikacin, isepamicin, gentamicin B, neomycin, paromomycin, lividomycin, ribostamycin, and butirosin aminoglycosides, as well as chloramphenicol and dalfopristin antibiotics. Assays were performed in a final volume of 250 μl in microtitre plates using a SpectraMAX PLUS spectrophotometer. A number of potential acylCoAs were also tested as substrates, including 500 μM acetyl-CoA, propionyl-CoA, or butyryl-CoA. Kinetic parameters for propionyl-CoA hydrolysis by the enzyme were determined by fitting rate data to Eq. 1., using Grafit version 4.0[24].

We also used a phosphocellulose binding assay to determine if any low levels of aminoglycoside acetylation activity could be detected. A select number of aminoglycosides at 0.5~1 mM concentrations were tested as potential substrates by incubation of Rv1347c protein and 300 μM [1-^{14}C]acetyl-CoA (65 μCi/mmol). Incubations proceeded for 30-minutes at room temperature prior to placement on P81 cation exchanger chromatography paper. Subsequent washing steps and determination of radioactivity retained on the paper were performed as previously described[25].

Protein Acetyltransferase Assays

To investigate whether the putative acetyltransferase possesses protein acetylation activity, we tested a number of available proteins as potential substrates, including H3/H4 histones type VIII-S, deoxyribonuclease, ribonuclease, MBP, lysozyme, lactic acid dehydrogenase, and APH(3′)-IIIa, using the procedure outlined previously[25]. The acetylation of H3/H4 histones by AAC(6′)-Ii as described[25] served as positive control.

Malonyl-CoA Decarboxylase Assays

Since the *Rv1347c* gene product also exhibits sequence homology to the *C*-terminal portion of a bacterial malonyl-CoA decarboxylase, we investigated the possibility that this protein may decarboxylate malonyl-CoA to produce CO_2 and acetyl-CoA. We therefore used a spectrophotometric assay to monitor the possible formation of acetyl-CoA by Rv1347c by coupling it to aminoglycoside acetylation by AAC(6′)-Ii. We also used reverse-phase HPLC to monitor any acetyl-CoA production and to confirm that HisRv1347c was not decarboxylating malonyl-CoA. Assays contained 35~70 μg of Rv1347c and 500 μM malonyl-CoA in 25 mM HEPES pH 7.5, 2 mM EDTA. Samples were incubated at room temperature for at least 1 hour prior to loading onto a Vydac 218TP54 RP column equilibrated with 97% 0.2 M sodium phosphate, pH 5.0 and 3% 0.2 M sodium phosphate, pH 5.0, 20% acetonitrile. Solutions and separation conditions were essentially as described previously[26], with modifications made to accommodate the use of a larger 25 cm×4.6 mm C18 column. Samples containing 500 μM CoA, acetyl-CoA, propionyl-CoA, butyryl-CoA, and malonyl-CoA, either alone or in combination, were used as controls and HPLC standards.

Antimicrobial Assay

Two hundred μl of overnight cultures of *E. coli*

BL21(DE3) containing either control or the various overexpression constructs described were plated onto LB agar supplemented with 100 μg/ml ampicillin. Filter paper disks (1/4″) were placed onto the plates, with or without and 20 μl of 100 μg/ml antibiotic solutions were added to each disk. The agar plates were incubated at 37°C overnight and the zones of inhibition measured. In a second set of experiments, a 100 mM IPTG solution was applied to the surface of the agar plates 1 hour before plating the *E. coli* cell cultures.

Minimum Inhibitory Concentration (MIC) Determinations

MICs of antibiotics against *E. coli* BL21(DE3) cells containing the described overexpression constructs were determined in liquid culture by serial dilution of a select number of aminoglycosides using standard methods[27].

Results

Protein Purification

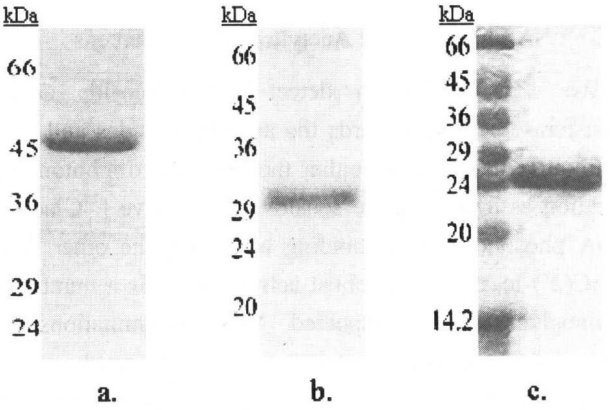
Overexpression of the putative aminoglycoside phosphotransferase and acetyltransferase enzymes in *E. coli* allowed us to obtain reasonable quantities of protein for purification and subsequent characterization. We ultimately utilized the His-tagged versions of all three proteins to ease purification, with subsequent overexpression and purification over Ni NTA agarose resulting in highly purified HisRv3817, HisRv3225c and HisRv1347c proteins (Fig. 1). This facilitated the investigation of these proteins as potential resistance enzymes, as described below.

Aminoglycoside Phosphotransferase Screens

HisRv3817 and HisRv3225c were first tested for the ability to phosphorylate a number of aminoglycosides by using the standard spectrophotometric PK/LDH assay. Although a large variety of aminoglycosides were tested, including representatives of all three classes of aminoglycosides, we were unable to detect any significant aminoglycoside kinase activity for either protein. We were, however, able to monitor significant ATP hydrolysis by both enzymes, where we determined HisRv3817 to have a K_M of 1.3 ± 0.3 mM and a k_{cat} of $0.020\pm0.002\,sec^{-1}$ for ATP hydrolysis. Thus, both proteins bind ATP and have low levels of ATPase activity.

As it was possible that the enzymes could modify aminoglycosides at very low levels that were masked by the level of ATP hydrolysis, we re-screened the aminoglycoside

Fig. 1. Purification of putative aminoglycoside modifying enzymes.



The overexpressed hexahistidine tagged proteins were purified using Ni-NTA agarose as described in Materials and Methods. a. 15% SDS-PAGE gel of purified HisRv3225c. b. 15% SDS-PAGE gel of purified HisRv3817. c. 20% SDS-PAGE gel of HisRv1347c. All gels were stained with Coomassie blue for visualization of proteins, with positions of molecular mass standards indicated to the left of each gel.

substrates using the more sensitive ^{32}P-ATP phosphocellulose binding assay. Using this method, we were able to monitor very minor aminoglycoside phosphorylation by HisRv3225c, although this activity was several hundred-fold lower than the activity observed for a typical aminoglycoside kinase, such as the enterococcal APH(2′)-Ia protein used as a positive control in these studies.

Additional Screens of Potential Kinase Substrates

As aminoglycoside kinases are structurally similar to eukaryotic protein kinases and have the ability to phosphorylate proteins/peptides on serine residues[23], we thought it was possible that the putative kinases might phosphorylate peptides. However, we were unable to detect any peptide or protein kinase activity for either putative kinase.

Considering the lack of any detectable aminoglycoside phosphorylation *in vitro*, it was not surprising that expression of the proteins in *E. coli* did not confer resistance to aminoglycosides and other antibiotics *in vivo*, as assessed by both filter disk assays and minimum inhibitory concentration determinations. The *N*-terminal hexahistidine tags did not appear to affect the ability of the

proteins to confer antibiotic resistance to the host, as the MICs of kanamycin A for *E. coli* expressing the non-tagged proteins or no protein was identical.

Aminoglycoside Acetyltransferase Screens

We were unable to detect aminoglycoside acetyltransferase activity towards the aminoglycosides and other antibiotics tested, using either the CoA spectrophotometric titration assay or a more sensitive radioactive [^{14}C]acetyl-CoA phosphocellulose binding assay. On the other hand, AAC(2′)-Ic, did show robust activity towards a number of aminoglycosides, as expected. MIC determinations and filter disk assays were consistent with these results, which indicated that no antibiotic resistance was conferred by the Rv1347c protein. Taken together, these results demonstrate that Rv1347c does not behave as an aminoglycoside or other antibiotic acetyltransferase enzyme both *in vitro* and *in vivo*.

Additional Screens of Potential Rv1347c Activities

Since Rv1347c also shows homology to the *C*-terminal region of a bacterial malonyl-CoA decarboxylase, we investigated whether this protein possesses decarboxylation activity. We did not detect any generation of acetyl-CoA upon incubation of the enzyme with malonyl-CoA and reverse-phase HPLC analysis confirmed that malonyl-CoA was not a substrate for Rv1347c.

As many aminoglycoside acetyltransferases are structurally similar to eukaryotic protein acetyltransferases and in some cases even demonstrate functional homology[25], we investigated whether Rv1347c could acetylate proteins. We screened various proteins as potential acetyl group acceptors and found that Rv1347c did not modify any of the proteins tested, suggesting that it does not function as a protein acetyltransferase.

To further investigate the possible function of this protein, we followed up on the observation that a low level of acetyl-CoA hydrolysis was evident both in the presence and absence of any potential antibiotic substrate. This prompted us to test several acylCoAs as substrates, including acetyl-CoA, propionyl-CoA, and butyryl-CoA. Thioesterase activity was demonstrated for all acyl-CoAs tested. Propionyl-CoA in particular was shown to be a relatively good substrate, with a K_M of 344±33 μM and a k_{cat} of 0.022±0.001 sec^{-1} determined for its hydrolysis by HisRv1347c. Thus, this protein possesses thioesterase activity with the capacity to discriminately bind and cleave different acylCoAs.

Discussion

M. tuberculosis, like numerous other bacteria, have in their genome unexpected genes that have the potential to encode antibiotic resistance proteins. Four predicted aminoglycoside resistance elements, two kinases and two acetyltransferases were identified in the sequencing of *M. tuberculosis* H37Rv. One of these, *aac*(2′)-*Ic*, has been well characterized biochemically as a functional aminoglycoside resistance gene[12], although it is unlikely that this is its primary function in the cell. An orthologue of this gene in *Providencia stuartii*, *aac*(2′)-*Ia*, has been demonstrated to encode an acetyltransferase potentially involved in peptidoglycan modification and its aminoglycoside antibiotic resistance ability has been suggested to be fortuitous[29]. As mycobacteria are sensitive to aminoglycosides inactivated by AAC(2′)[11], it is therefore likely as well that antibiotic resistance is not a primary function of this enzyme.

The second putative aminoglycoside 6′-acetyltransferase is derived from the orf *Rv1347c*. Sequence analysis reveals homology to AAC(6′) enzymes and other members of the larger GCN5 acyltransferase superfamily. Our results conclusively demonstrate that this enzyme does not recognize aminoglycoside or a selected group of potential protein substrates that are very frequently acylated by members of the GCN5 superfamily. Additionally, Rv1347c shows homology to a malonyl-CoA decarboxylase from *Saccharopolyspora erythraea*, however here again we failed to conclusively demonstrate this activity *in vitro*. Acyltransferases and malonyl-CoA decarboxylase discriminately bind various acylCoAs, and we noted that Rv1347c does in fact recognize acylCoAs and utilizes them as substrates in a thioesterase function. Thus Rv1347c is functionally annotated as an acylCoA thioesterase with preference for propionyl-CoA. *Rv1347c* is flanked by numerous ORFs that show homology to genes involved in polyketide synthesis and assembly, particularly the *Rv1344*, *fadD33*, *fadE14* genes. *Rv1347c* may therefore encode an enzyme that acts in a chain releasing function during biosynthesis of an as yet uncharacterized metabolite.

Characterization of the two putative aminoglycoside kinases, Rv3817 and Rv3225c also demonstrated that these predicted aminoglycoside resistance genes do not in fact show this activity in biochemical and microbiological assays. The 51 kDa Rv3225c did show modest aminoglycoside kinase activity in a highly sensitive radioassay, however the activity while reproducible, was not robust in comparison to a well characterized positive control. Both enzymes also demonstrated ATPase activity, which may be significant, but we cannot rule out the role of contaminating

phosphatases. Representative substrates of the common subclasses of kinases also failed to show significant activity. Therefore the true role of these enzymes remains a mystery. Unlike the Rv1347c thioesterase, their positioning within the genome reveals no obvious clues to their activity.

The results of this study have demonstrated that the putative aminoglycoside modifying enzymes Rv1374c, Rv3817c and Rv3225c do not behave as antibiotic resistance enzymes under our experimental conditions, and that AAC(2′)-Ic does demonstrate the predicted activity as previously shown. These results reinforce the necessity of biochemical assays to follow up the *in silico* annotation phase of microbial genome analysis. More importantly however, the analysis of these proteins reveals the large number of microbial genes with the capacity to evolve into resistance elements. All of the gene products studied bound the appropriate cosubstrates acylCoA or ATP as evidenced by their catalytic properties (thioesterase or ATPase). While somewhat paradoxically, aminoglycoside resistance in *M. tuberculosis* seems to be exclusively the result of target ribosomal mutations[30,31]. The modest activity of these gene products suggests the potential for selection of the emergence of upregulated AAC(2′)-Ic production (analogous to the *P. stuartii* phenomenon[32]) or altered Rv3225c with increased aminoglycoside kinase activity resulting in resistance. This potential, embedded in the genetic background of this organism should be considered when alternate therapies for infection are considered that may involve aminoglycoside use.

Acknowledgments

This research was supported by grant MT-13536 from the Canadian Institutes of Health Research. GDW is the recipient of a Canada Research Chair in Biochemistry.

References

1) Pallen, M. J.: Microbial genomes. Mol. Microbiol. 32: 907~912, 1999
2) Fraser, C. M.; J. A. Eisen & S. L. Salzberg: Microbial genome sequencing. Nature 406: 799~803, 2000
3) Costa; Y., M, Galimand, R. Leclercq, J. Duval & P. Courvalin: Characterization of the chromosomal *aac*(6′)-*Ii* gene specific for *Enterococcus faecium.* Antimicrob. Agents Chemother. 37: 1896~1903, 1993
4) Cole, S. T.; K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, K. Mungall, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. M. Davies, K. Devlin, S. Duthoy, T. Feltwell, A. Fraser, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, C. Lacroix, J. Maclean, S. Moule, L. Murphy, K. Oliver, M. A. Quail, M. A. Rajandream, K. M. Rutherford, S. Rutter, K. Seeger, S. Simon, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, K. Taylor, S. Whitehead, J. R. Woodward & B. G. Barrell: Massive gene decay in the leprosy bacillus. Nature 409: 1007~1011, 2001
5) Wright, G. D.: Aminoglycoside-modifying enzymes. Curr. Opin. Microbiol. 2: 499~503, 1999
6) Udou, T.; Y. Mizuguchi & T. Yamada: Biochemical mechanisms of antibiotic resistance in a clinical isolate of *Mycobacterium fortuitum.* Presence of β-lactamase and aminoglycoside-acetyltransferase and possible participation of altered drug transport on the resistance mechanism. Am. Rev. Respir. Dis. 133: 653~657, 1986
7) Udou, T.; Y. Mizuguchi & R. J. Wallace, Jr.: Patterns and distribution of aminoglycoside-acetylating enzymes in rapidly growing mycobacteria. Am. Rev. Respir. Dis. 136: 338~343, 1987
8) Wallace, R. J. Jr.; S. I. Hull, D. G. Bobey, K. E. Price, J. M. Swenson, L. C. Steele & L. Christensen: Mutational resistance as the mechanism of acquired drug resistance to aminoglycosides and antibacterial agents in *Mycobacterium fortuitum* and *Mycobacterium chelonei.* Evidence is based on plasmid analysis, mutational frequencies, and aminoglycoside-modifying enzyme assays. Am. Rev. Respir. Dis. 132: 409~416, 1985
9) Hull, S. I.; R. J. Wallace, Jr., D. G. Bobey, K. E. Price, R. A. Goodhines, J. M. Swenson & V. A. Silcox: Presence of aminoglycoside acetyltransferase and plasmids in *Mycobacterium fortuitum.* Lack of correlation with intrinsic aminoglycoside resistance. Am. Rev. Respir. Dis. 129: 614~618, 1984
10) Ho, Ii; C. Y. Chan & A. F. Cheng: Aminoglycoside resistance in *Mycobacterium kansasii*, *Mycobacterium avium-M. intracellulare*, and *Mycobacterium fortuitum*: are aminoglycoside-modifying enzymes responsible? Antimicrob. Agents Chemother. 44: 39~42, 2000
11) Aínsa, J. A.; E. Perez, V. Pelicic, F. X. Berthet, B. Gicquel & C. Martin: Aminoglycoside 2′-*N*-acetyltransferase genes are universally present in mycobacteria: characterization of the *aac*(2′)-*Ic* gene from *Mycobacterium tuberculosis* and the *aac*(2′)-*Id* gene from *Mycobacterium smegmatis.* Mol. Microbiol. 24: 431~441, 1997
12) Hegde, S. S.; F. Javid-Majd & J. S. Blanchard: Overexpression and mechanistic analysis of chromosomally encoded aminoglycoside 2′-*N*-acetyltransferase [AAC(2′)-Ic] from *Mycobacterium tuberculosis.* J. Biol. Chem., 276: 45876~45881, 2001
13) Cole, S. T.; R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, *et al.*: Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393: 537~544, 1998
14) Thompson, C. J. & G. S. Gray: Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferases encoded by resistance plasmids. Proc. Natl. Acad. Sci. USA 80: 5190~5194, 1983
15) Salauze, D. & J. Davies: Isolation and characterization

of an aminoglycoside phosphotransferase from neomycin-producing *Micromonospora chalcea*; comparison with that of *Streptomyces fradiae* and other producers of 4,6-disubstituted 2-deoxystreptamine antibiotics. J. Antibiotics 44: 1432~1443, 1991

16) HACHLER, H.; P. SANTANAM & F. H. KAYSER: Sequence and characterization of a novel chromosomal aminoglycoside phosphotransferase gene, *aph*(3′)-*IIb*, in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 40: 1254~1256, 1996

17) PANSEGRAU, W.; L. MIELE, R. LURZ & E. LANKA: Nucleotide sequence of the kanamycin resistance determinant of plasmid RP4: homology to other aminoglycoside 3′-phosphotransferases. Plasmid 18: 193~204, 1987

18) SHAW, K. J.; C. A. CRAMER, M. RIZZO, R. MIERZWA, K. GEWAIN, G. H. MILLER & R. S. HARE: Isolation, characterization, and DNA sequence analysis of an AAC(6′)-II gene from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 33: 2052~2062, 1989

19) TORIYA, M.; M. SAKAKIBARA, K. MATSUSHITA & T. MOROHOSHI: Nucleotide sequence of aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)] determinant from *Serratia* sp. 45. Chem. Pharm. Bull. 40: 2473~2477, 1992

20) HSIEH, Y. J. & P. E. KOLATTUKUDY: Inhibition of erythromycin synthesis by disruption of malonyl-coenzyme A decarboxylase gene *eryM* in *Saccharopolyspora erythraea*. J Bacteriol. 176: 714~724, 1994

21) BOEHR, D. D.; W. S. LANE & G. D. WRIGHT: Active site labeling of the gentamicin resistance enzyme AAC(6′)-APH(2″) by the lipid kinase inhibitor wortmannin. Chem. Biol. 8: 791~800, 2001

22) WRIGHT, G. D. & P. LADAK: Overexpression and characterization of the chromosomal aminoglycoside 6′-*N*-acetyltransferase from *Enterococcus faecium*. Antimicrob. Agents Chemother. 41: 956~960, 1997

23) DAIGLE, D. M.; G. A. MCKAY, P. R. THOMPSON & G. D. WRIGHT: Aminoglycoside antibiotic phosphotransferases are also serine protein kinases. Chem. Biol. 6: 11~18, 1999

24) LEATHERBARROW, R. J.: Grafit Ver. 4.0.12. Erithacus Software LTD, Staines UK, 2000

25) WYBENGA-GROOT, L. E.; K. DRAKER, G. D. WRIGHT & A. M. BERGHUIS: Crystal structure of an aminoglycoside 6′-*N*-acetyltransferase: defining the GCN5-related *N*-acetyltransferase superfamily fold. Structure Fold Des. 7: 497~507, 1999

26) KING, M. T. & P. D. REISS: Separation and measurement of short-chain coenzyme-A compounds in rat liver by reversed-phase high-performance liquid chromatography. Anal. Biochem. 146: 173~179, 1985

27) National Committee for Clinical Laboratory Standards: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed Approved standard M7-44, 1997

28) NOGUCHI, N.; A. EMURA, H. MATSUYAMA, K. O'HARA, M. SASATSU & M. KONO: Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2′-phosphotransferase-I in *Escherichia coli*. Antimicrob. Agents Chemother. 39: 2359~2363, 1995

29) FRANKLIN, K. & A. J. CLARKE: Overexpression and characterization of the chromosomal aminoglycoside 2′-*N*-acetyltransferase of *Providencia stuartii*. Antimicrob. Agents Chemother. 45: 2238~2244, 2001

30) RAMASWAMY, S. & J. M. MUSSER: Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber. Lung Dis. 79: 3~29, 1998

31) GILLESPIE, S. H.: Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. Antimicrob. Agents Chemother. 46: 267~274, 2002

32) RATHER, P. N.; E. OROSZ, K. J. SHAW, R. HARE & G. MILLER: Characterization and transcriptional regulation of the 2′-*N*-acetyltransferase gene from *Providencia stuartii*. J. Bacteriol. 175: 6492~6498, 1993

33) ALTSCHUL, S. F.; T. L. MADDEN, A. A. SCHAFFER, J. ZHANG, Z. ZHANG, W. MILLER & D. J. LIPMAN: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389~3402, 1997

34) HIGGINS, D. G. & P. M. SHARP: Fast and sensitive multiple sequence alignments on a microcomputer. Comput. Appl. Biosci. 5: 151~153, 1989