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Production of aminoglycosides in non-aminoglycoside producing *Streptomyces lividans* TK24

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Abstract—The pRBM4 cosmid, which harbors a putative cluster of genes spanning a 31.8-kb chromosomal region of the ribostamycin producer *Streptomyces ribosidificus* ATCC 21294, was heterologously expressed in *Streptomyces lividans* TK24. ESI-MS/MS, HPLC, and LC-ESI MS analyses showed that the transformation gave rise to ribostamycin production in various culture broths. This is the first report of heterologous aminoglycoside production.

More than two-thirds of the naturally occurring, clinically useful antibiotics have been isolated from members of the genus *Streptomyces*.[1] Among these, the aminoglycosides constitute an important group of antibiotics, which have been shown to be particularly useful in the treatment of infections caused by virulent Gram-negative pathogens, for which therapeutic options are currently limited.[2] Tobramycin and neomycin are typical natural aminoglycosides, and are extensively used in solutions or ointments for the treatment of various skin and eye infections. However, the emergence and rapid spread of aminoglycoside resistance among human pathogens poses a serious threat to human health and has provided a potent impetus for the development of novel antibiotics that circumvent current resistance mechanisms. The genetic manipulation of both, existing and new, biosynthetic pathways for the incorporation of structural modifications is particularly interesting to aminoglycoside research, as it provides an alternative to complicated chemical syntheses. Detailed functional analysis of the relevant biosynthetic genes is, however, a prerequisite for such approaches.

Structurally, the majority of the aminoglycosides share a common aglycon, 2-deoxystreptamine (DOS), which is linked with other sugar subunits via *O*-glycosidic linkages. The 4- and 5-hydroxyl groups of DOS, for example, are glycosylated in ribostamycin (Rbm) (Fig. 4), neomycin (Nem), and butirosin (Bn); the 4- and 6- hydroxyl groups are glycosylated in tobramycin (Tbm), gentamicin (Gm), and kanamycin (Km); and the 5-hydroxyl group is glycosylated in apramycin and hygromycin B. Although the biosynthesis of these antibiotics had been studied few decades ago,[3–6] genetic and biochemical studies addressing the formation of these compounds have recently begun with the isolation of the Bn, Gm, Tbm, Km, Nem, and Rbm biosynthetic gene clusters.[7–11] These efforts are, however, presently limited to the characterization of only a few of the DOS biosynthetic enzymes (DOI synthase, L-glutamine: DOI-aminotransferase and dehydrogenase), which, in this case, are nucleotidyltransferase and resistance genes.[7,9,10,12–18] More importantly, none of these previous studies has thus far functionally demarcated the boundaries of the gene clusters, and as a result, the biosynthetic pathways remain obscure.

Neomycin, ribostamycin, and butirosin are architecturally simple and share three common subunits: neosamine, DOS, and ribose. The identification and characterization of biosynthetic genes for either one of

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Figure 1. Antibacterial assay of isolated compounds and authentic ribostamycin against *B. subtilis*: P, *S. lividans* TK24/pOJ446 metabolites (20 μl); T, *S. lividans* TK24/pRMB4 metabolites (20 μl); W, crude ribostamycin from *S. ribosidificus* (40 μl); S, authentic ribostamycin (Sigma, St. Louis, MO) (50 μg). Samples were isolated under identical condition and amount.

these compounds would provide a framework for further investigation of related members with more complicated structures. Taking this hypothesis into account, we have previously cloned a putative Rbm biosynthesis associated gene cluster from *Streptomyces ribosidificus*[11] (see supporting information, Fig. 1S) and characterized a key DOS biosynthetic enzyme (RbmA) as well as an Rbm-inactivating enzyme (RbmI).[11] In this study, we report the expression of the entire Rbm gene cluster in non-aminoglycoside producing *Streptomyces lividans* TK24, as well as the isolation and characterization of Rbm extracted from that strain.

A cosmid library of *S. ribosidificus* genomic DNA was generated using the *Streptomyces-Escherichia coli* shuttle vector pOJ446. This vector harbors *oriT* sequences and an apramycin resistance marker (*aac(3)IV*), facilitating pUZ8002-mediated conjugal transformation into *Streptomyces* and transformant selection, respectively.[1] A cosmid designated pRBM4 was isolated via phase-wise screening of the library. This cosmid harbors a putative Rbm biosynthetic gene cluster flanked by the Rbm resistance genes acetyltransferase (*rbmI*) and phosphotransferase (*rph*)[11] (see supporting information, Fig. 1S and EMBL nucleotide database accession number AJ748131). The cosmid was transformed into *E. coli* ET12567/pUZ8002 competent cells according to standard procedures.[19] The resulting ET transformant was then conjugated with the spores of *S. lividans* TK24 on MS agar plate, again following established protocols.[1,19] Overlaying each conjugation plate after 12 h with water (1 ml) containing 0.5 mg nalidixic acid and 1 mg aparamycin yielded several exconjugants, which were designated as *S. lividans*/pRBM4. In a similar fashion,

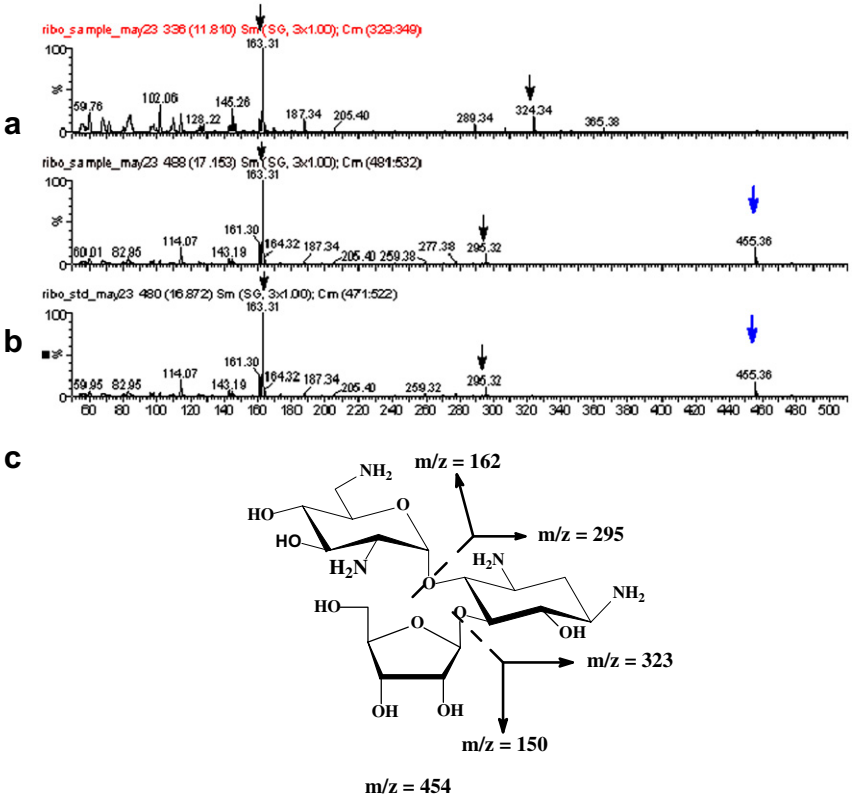

Figure 2. ESI (+)-MS/MS spectrum of isolated compound and authentic ribostamycin (Sigma, St. Louis, MO). (a) compounds isolated from *S. lividans*/pRBM4. (b) Authentic ribostamycin. Arrow indicates the ribostamycin (455) and ion products (324, 295, 163) formed from ribostamycin. (c) Fragmentation scheme proposed for ribostamycin.

pOJ446 was also transformed, yielding *S. lividans*/pOJ446. The transformants were further verified by re-isolating pRBM4 and pOJ446 from *S. lividans*/pRBM4 and *S. lividans*/pOJ446, and conducting restriction analyses.[1,19]

For the analytical scale steps, each of the strains was cultured in 2 L (100 ml × 20 baffled flasks) of R2YE medium for 5 days at 28 °C. The pH of the broth was adjusted to 2, and the mycelial cake removed via filtration. The filtrate was then neutralized and passed through Amberlite IRC 50 ion-exchange resin (Arcos, USA). The resin was washed with 10 volumes of distilled water, which removed the majority of the unbound impurities. Finally, the crude antibiotic was eluted using 2 N ammonium hydroxide. The pH of the effluent was adjusted to 4.5 with sulfuric acid and the crude ribostamycin (5 mg with 80% purity) precipitated as a sulfate salt using ice-cold methanol.

Thin-layer chromatography (TLC) analyses revealed that Rbm was a major compound in the crude fraction obtained from the *S. lividans*/pRBM4 culture broth, whereas no Rbm was detected in *S. lividans*/pOJ446 cultures (see supporting information, Fig. 2S). Furthermore, the fraction from the former strain displayed marked activity against *Bacillus subtilis*, while the fraction obtained from the latter strain had no antibiotic activity (Fig. 1). Subsequently, the analysis of isolated compounds by ESI-MS (Data not shown) and ESI-MS/MS (Fig. 2) showed that ribostamycin was produced by *S. lividans*/pRBM4. In order to confirm, the active fraction contained Rbm, UV-visible derivatives of the fraction and standard Rbm (Sigma, St. Louis, MO) were prepared in accordance with a previous report by Stead and Richards,[20] with slight modifications, after which high-performance liquid chromatography (HPLC) and electro spray ionization-mass (ESI-MS) profiles were obtained and compared. An aqueous solution of Rbm (Sigma, 1 mg/ml) was combined with 20 μl of 9-fluorenylmethyl chloroformate (FMOCl, 20 μg/mL) and incubated for 1 h at 37 °C. HPLC analyses of the products were conducted at 260 nm using a C-18 column (Mightysil RP-18 Gp, Japan) with a linear acidified (0.1% trifluoroacetic acid) water-to-acetonitrile gradient from 100% to 0%.

Four peaks were detected in the HPLC chromatogram for the derivatives of standard Rbm. Identical sets of peaks were also observed with the products of *S. lividans*/

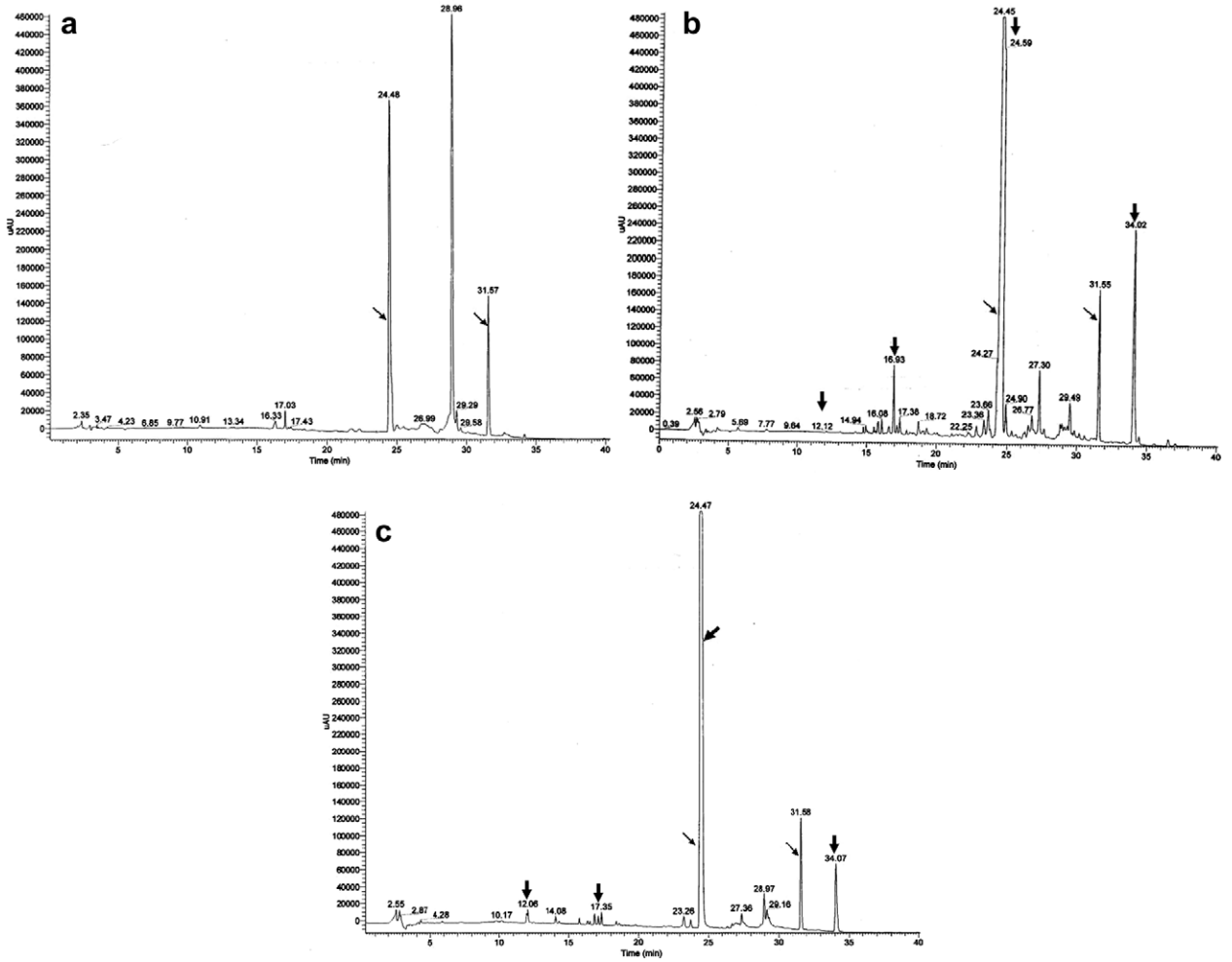

Figure 3. HPLC analysis of FMOCl-derivatized compounds: (a) metabolite derivatives of *S. lividans* TK24/pOJ446; (b) *S. lividans* TK24/pRMB4; (c) Standard ribostamycin. The thick and thin arrows point at the peaks for the derivatives of ribostamycin and other impurities, respectively.

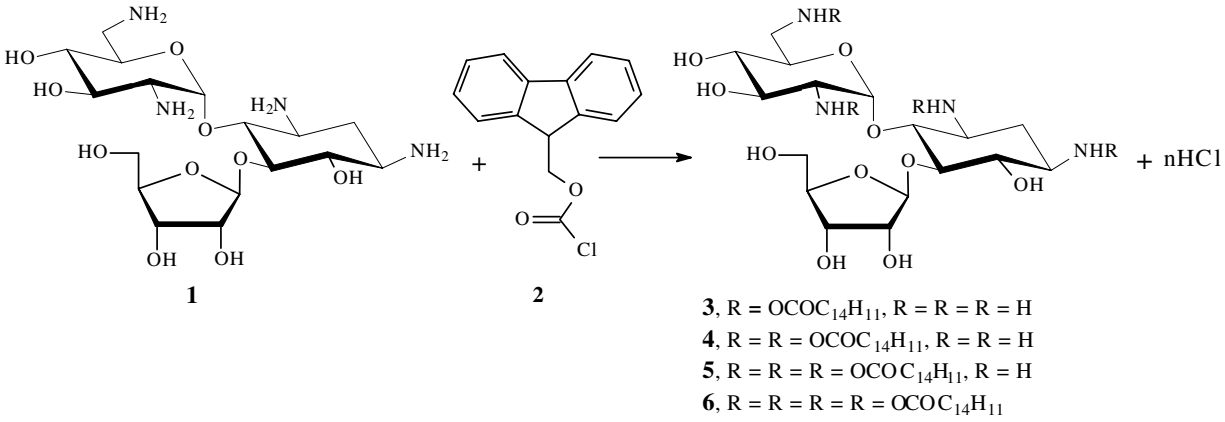

Figure 4. Reaction of ribostamycin [**1**] with 9-fluorenylmethyl chloroformate (FMOCl) [**2**]. The proposed derivatives **3**, **4**, **5**, **6** correspond to the derivatization of one, two, three, and four amino groups of Rbm, respectively, and the number (*n*) may vary from 1 to 4.

pRBM4. However, no single corresponding peak was detected with the products of the reference strain, *S. lividans*/pOJ446. These four compounds may have been the result of an incomplete derivatization of the four amino groups of Rbm. In order to verify this, both derivatized samples were subjected to LC-ESI MS analysis, which revealed an identical set of four peaks at 12.09, 16.9, 24.5, and 34.07 min (Fig. 3), corresponding to molecular masses of 677, 899, 1121, and 1360, respectively (see supporting information, Fig. 3S(B) and (C)). The first three compounds corresponded to the $[M+H]^+$ for one, two, and three amide derivatives (products of amino group reactions with FMOCl) of Rbm (Fig. 4), whereas the fourth was found to correspond to the $[M+H_2O]^+$ of a fully derivatized Rbm molecule. The lack of all of these compounds in identically derivatized products of the control strain, *S. lividans*/pOJ446 (Fig. 3a) and supporting information (Fig. 3S(A)), clearly demonstrated that the plasmid alone exerted no significant influence on the metabolite production pattern, while pRBM4 clearly stimulated Rbm production in *S. lividans* TK24.

In summary, the pRBM4 contains all the genes whose corresponding enzymes are likely to be involved in the intriguing steps of Rbm biosynthesis. Expression of the cosmid in *S. lividan*s affording Rbm in comparable amount to that of wild type strain not only provides unambiguous assignment of genetic boundary for Rbm biosynthesis but also paves the way for the further biochemical analyses of Rbm biosynthetic enzymes. This study is the first to report on a biosynthetic gene cluster expression giving rise to aminoglycoside antibiotic production in a non-producing heterologous host. The work described in this study may serve as a foundation for further studies on Rbm biosynthesis and other aminoglycosides, and possibly aid future development of improved Rbm-type drugs via combinatorial biosynthesis protocols.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.035.

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