Puromycin A, B and C, cryptic nucleosides identified from *Streptomyces alboniger* NRRL B-1832 by PPtase-based activation

Xiaoli Yan a,1, Benyin Zhang b,1, Wenyia Tian a, Qi Dai a, Xiaqin Zheng a, Ke Hu a, Xinxin Liu a, Zixin Deng a, Xudong Qu a, c,*

a Key Laboratory of Combinatorial Biosynthesis and Drug Discovery Ministry of Education, School of Pharmaceutical Sciences, Wuhan University, China
b State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, China
c Jiangshu National Synergetic Innovation Center for Advanced Materials (SICAM), China

1 These authors contributed equally.

**A B S T R A C T**

Natural product discovery is pivot for drug development, however, this endeavor is often challenged by the wide inactivation or silence of natural products biosynthetic pathways. We recently developed a highly efficient approach to activate cryptic/silenced biosynthetic pathways through augmentation of the phosphopantetheinylation of carrier proteins. By applying this approach in the *Streptomyces alboniger* NRRL B-1832, we herein identified three cryptic nucleosides products, including one known puromycin A and two new derivatives (puromycin B and C). The biosynthesis of these products doesn’t require the involvement of carrier protein, indicating the phosphopantetheinyl transferase (PPtase) indeed plays a fundamental regulatory role in metabolites biosynthesis. These results demonstrate that the PPtase-based approach have a much broader effective scope than the previously assumed carrier protein-involving pathways, which will benefit future natural products discovery and biosynthetic studies.

Natural products are a major source for drug discovery and development. It is revealed that more than 50% small molecule drugs (including pesticides) are from natural products or their derivatives [1,2]. Although the discovery of natural products is pivot for drug development, the rate of identification of useful molecules has been steadily declined since from the late of last century [2,3]. One of major reason for that is the widely inactivation and silence of microbial biosynthetic pathways [2,4]. It is estimated that more than 50% of microbial metabolites in the genome are indeed not encoded under normal laboratory culturing conditions due to the absence of essential regulation signals to trigger their biosynthesis [5]. Metabolites biosynthesis depends on orchestrated regulations of biomachines involving translation from DNA to working proteins [6]. The inactivation or silence of natural products biosynthesis is majorly resulted by missing or improper regulations during that process [2,4–6].

So far a few strategies targeting on altering epigenetic (eukaryote only) [7,8], transcriptional [9] and translational regulation [10,11] as well as elicitation [12–14] have been developed to successfully activate natural products biosynthesis. Very recently, we developed an additional approach from the aspect of protein-modification level regulation to awake cryptic/silenced biosynthetic pathways [15]. Biosynthesis of polyketide (PK), nonribosomal peptide (NRP) and fatty acid (FA) as well as a few primary metabolites including lysine and tetrahydrofolate are depended on a conserved protein modification that requires phosphopantetheinylation transferase (PPtase) to convert carrier proteins (CPs) from inactive apo-form into active holo-form [16]. By augmentation of the phosphopantetheinylation in vivo, we observed a significantly high number of strains (70%) from 33 tested Actinomycetes produce activated metabolites [15]. Isolation of activated products from two strains resulted to successfully identify two groups of cryptic polyketide products, confirming its high efficiency to activate CP involving metabolites [15]. By continued application of this approach, we herein confirmed that the effective scope of this

https://doi.org/10.1016/j.synbio.2018.02.001
2405-805X/© 2018 The Authors. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
PPtase-activation approach can be indeed extended to non-CP involving pathways. Overexpression of the broad-selective PPTases into the *Streptomyces alboniger* NRRL B-1832 resulted in activation and identification of three nucleosides products puromycins (A-C) whose biosynthesis doesn't require involvement of CP. This result demonstrates the very broad-utility of this approach which will benefit further natural products discovery and biosynthetic studies.

2. Material and methods

2.1. General experimental details

NMR spectra were measured on a Bruker Avance DRX-600 spectrometer (1H: 600 MHz, 13C: 150 MHz). HPLC Analysis was carried out on a SHIMADZU LC-20A Prominance HPLC system with a column of Diamonsil (C18 (2), 5 μm, 250 × 4.6 mm, Dikma Technologies Inc.). Semipreparative HPLC was performed with a ZORBAX SB-C18 5 μm column (9.4 × 250 mm); HPLC-MS analysis was carried out on a Thermo Instruments HPLC system connected to LCQ Fleet electrospray ionization (ESI) mass spectrometer (ThermoFisher Scientific Inc.). HPLC-ESI-high resolution MS (HPLC-ESI-HRMS) analysis was carried out on ESI-LTQ Orbitrap (ThermoFisher Scientific Inc.). Sephadex LH-20 (25–100 μm, Pharmacia Biotek, Denmark) was used for column chromatography. All solvents used were analytical or HPLC grade. *S. alboniger* was cultivated either on the MS agar (agar 2 g, mannitol 2 g, soya water) or tryptic soy broth (TSB) for growth.

2.2. Construction of *S. alboniger* NRRL B-1832-PPTase and the control strain

Following the previous developed procedure, the empty vector pIB139 and pWHU2449 [15] which carries the gene cassette *ermE*-sfp-svp were introduced into *S. alboniger* NRRL B-1832 through conjugation to yield the control strain and PPTase overexpression strains, respectively [15].

2.3. Fermentation and metabolites profile analysis

*S. alboniger* NRRL B-1832 and recombinant strain *S. alboniger* NRRL B-1832-PPTase were cultured in media TSB for 2 days, then inoculated in IWL-4 medium (soluble starch 1 g, K2HPO4 0.1 g, MgSO4 0.1 g, NaCl 0.1 g, (NH4)2SO4 0.2 g, CaCO3 0.2 g, FeSO4 0.1 mg, MnCl2-6H2O 0.1 mg, ZnSO4 0.1 mg, yeast extract 5.0 mg, Tryptone 100 mg, in 100 mL ddH2O, pH = 7.2; yeast extract and tryptone were purchased from Angel Yeast Co. Ltd.) and cultured for 5 days at 28 °C. After fermentation, the liquid culture broth was extracted with the equal volume ethyl acetate under sonication for 20 min. Organic phase was transferred and dried by vacuum at low temperature (30 °C). Metabolites were subsequently redissolved by 1 mL ethanol and filtrated by a 0.22 μm membrane to remove particles before HPLC or HPLC-MS analysis. For sample analysis, a SHIMADZU LC-20A Prominance HPLC system was used. The flow-rate was 1 mL min⁻¹ and the column temperature was maintained at 25 °C. The mobile phase consisted of a mixture of acetonitrile (B) and 5 mM NH4Ac aqueous (A). The gradient elution was employed as follows: 5–100% (v/v) B at 0–30 min; 100% B at 30–33 min; 100–5% B at 33–34 min; 5% B at 34–40 min.

2.4. Cytotoxic activity assay

Two human cancer cell lines (HL60, NB4) were seeded at a density of 3–5 × 10³ per well in 96-well plate and incubated overnight, and then treated with compounds 1–3 in various concentrations, solvent DMSO (<0.1%) was used as a negative control, doxorubicin (2 μM) was used as a positive control. After 48 h treatment, the viability was determined using a CCK-8 kit according to the manufacturer’s instructions. The 50% inhibiting concentration (IC50, μM) was calculated by SPSS software version 13.0.

3. Results

3.1. Activation and isolation of metabolites in *S. alboniger* NRRL B-1832

To activate metabolites in the *S. alboniger* NRRL B-1832, two broad-selective PPTase genes, sfp and svp from the *Bacillus subtilis* and *S. verticillus* respectively [15] were introduced in a way of *ermE*-sfp-svp (*ermE* is a strong constitutive promoter) into the strain through conjugation (see 2.2). Analysis of the fermentation broth of the wild-type, control strain which has an empty pIB139 integrated in the genome, and PPTase strains revealed that a number of new peaks were activated and produced by over-expression of the PPTase genes (Fig. 1b). To isolate the major products, a large scale fermentation was employed. NRRL B-1832-PPTase was cultured in IWL-4 medium (100 × 100 mL) within 300 mL baffled shake flasks, each containing a loop of a 20 cm spring (diameter 1 cm) at 28 °C and 200 rpm for 5 days. The culture broth was extracted three times with equal volume ethyl acetate at room temperature. The filtrate was evaporated in vacuo to obtain crude extract, which was further subjected to Sephadex LH-20 gel column (20 × 1800 mm) separation and eluted successively with methanol. The eluted fractions containing compounds 1 (6.3 mg), 2 (3.3 mg) and 3 (4.2 mg) were combined and semi-prepared on an SHIMADZU LC-20A Prominance HPLC system using Agilent ZORBAX SB-C18 (5 μm, 250 × 9.4 mm, Agilent Technologies Inc.) at a flow rate of 3 mL min⁻¹ over a 40 min gradient program with 5 mM NH4Ac as eluent A.

3.2. Structural characterization of puromycin A-C

Compound 1 was obtained as a white amorphous powder. Interpretation of the 1H, 13C NMR, HSQC, HMBC and COSY spectroscopic data, and ESI-HRMS data showed compound 1 was puromycin [17], which is an aminonucleoside antibiotic reported in 1952 [18]. It is a broad spectrum secondary metabolite active against Gram-positive bacteria, protozoans, and mammalian cells, including tumor cells [19] by inhibiting both 70S and 80S ribosomes [20].

Compound 2 was obtained as a white amorphous powder. The 1H and 13C NMR spectrum of 2 are similar to those of 1, showed the presence of structural skeleton of puromycin. However, in the 1H NMR spectrum of 2, there appears a methyl signal at δH 1.95, meanwhile, two carbon signal at δC 21.8 and 170.9 appears in the 13C NMR spectrum. The HMBC correlations of proton at δH 1.95 to carbons at δC 54.9 and 170.9 suggested the presence of an acetyl group linked at amino of tyrosine group. On the basis of these data and ESI-HRMS data m/z 514.2432 [M + H]+ (calcd for 514.2409, C22H23N6O5), the compound 2 was shown in Fig. 1a and named puromycin B (Fig. 1a and Table 1).

Compound 3 was obtained as a white amorphous powder. ESI-HRMS data showed the mass 556.2544 [M + H]+ (calcd for 556.2514, C20H24N6O5). The molecular weight of 3 is 43 greater than 2, suggesting it bears an additional acetyl group, which is also revealed from the comparison to 1H NMR and 13C NMR spectrum of 2, there appear additional signals for methyl group at δH 1.99 and two carbons at δC 20.1 and 169.6. We thereby confirmed that another acetyl group was linked to the glycosyl group by the HMBC
Fig. 1. Activation of puromycin biosynthesis in S. alboniger NRRL B-1832 by overexpression of the PPtases. (a) Structures of compounds 1–3 and key $^1$H–$^1$H COSY and HMBC correlations for 2 and 3, (b) HPLC traces show the metabolites profiles in the PPtase strain (I), control strain (II) and wild-type strain (III).

Table 1
NMR data of compounds 2 and 3 in a mixed solvent of DMSO-$d_6$ and CD$_3$OD-$d_4$ (ration is 1:1).

| Position | 2 $\delta_H$ (multi, $J$ in Hz) | $\delta_C$ | 3 $\delta_H$ (multi, $J$ in Hz) | $\delta_C$ |
|----------|-------------------------------|------------|-------------------------------|------------|
| 2        | 8.28, s                       | 152.0      | 8.30, s                       | 152.2      |
| 4        | 140.8                         | 150.0      | 120.3                         | 120.0      |
| 5        | 120.3                         | 154.9      | 6-N-(CH$_3$)$_2$ 3.56, brs     | 39.7       |
| 6        | 154.9                         |            | 172.4                         |            |
| 8        | 8.41, s                       | 138.0      | 8.18, s                       | 138.2      |
| 6-N-(CH$_3$)$_2$ | 3.56, brs           | 37.9       | 5.16, d (3.1)                 | 87.0       |
| 1'       | 6.05, d (3.1)                 | 90.3       | 6.05, d (3.1)                 | 74.3       |
| 2'       | 4.63–4.65, m                  | 73.7       | 4.73, t (6.7)                 | 74.3       |
| 3'       | 4.60–4.62, m                  | 50.9       | 4.48–4.50, m                  | 49.4       |
| 4'       | 4.05, ddd (7.2, 3.4, 2.3)     | 83.7       | 3.93 ddd (7.2, 3.4, 2.3)      | 83.6       |
| 5'       | 3.84, dd (12.4, 2.2)          | 61.2       | 3.62, dd (12.4, 2.2);         | 61.2       |
| 6'       | 3.62, dd (12.4, 3.5)          | 61.2       | 3.44, dd (12.4, 3.5)          | 61.2       |
| 1''      |                                | 172.4      |                               | 172.0      |
| 2''      | 4.68, dd (8.4, 6.5)           | 55.0       | 4.49, dd (8.4, 6.5)           | 54.9       |
| 3''      | 3.07, dd (13.8, 6.5)          | 37.3       | 2.87, dd (13.8, 6.5)          | 37.1       |
| 4''      | 2.91, dd (13.8, 8.4)          |            | 2.71, dd (13.8, 6.5)          |            |
| 5'', 9'' | 7.26, d (8.4)                 |            | 7.13, d (8.4)                 |            |
| 6'', 8'' | 6.93, d (8.4)                 |            | 6.80, d (8.4)                 |            |
| 7''      | 158.6                         |            | 158.4                         |            |
| 7''-O-CH$_3$ | 3.83, s                        | 54.8       | 3.80, s                       | 54.5       |
| 2''-NH-CO |                                | 170.9      |                               | 170.0      |
| 2''-NH-CO-CH$_3$ | 1.95, s                     | 21.8       | 1.77, s                       | 22.1       |
| 2'-O-CO  |                                |            |                               |            |
| 2'-O-CO-CH$_3$ |                              | 1.99, s                  | 20.1       |
correlations from the proton at δC 1.99 to the carbons at δC 169.6 and δC 74.3. Thus, the compound 3 was shown in Fig. 1a and named puromycin C (Fig. 1a and Table 1).

2.3. Bioactivity assay of puromycin A-C

All of the isolated compounds were further tested for their cytotoxicity against two human cancer cell lines (HL60, NB4) (Table 2). It was observed that compound 1 exhibited powerful cytotoxic activity to HL60 and NB4 with IC50 values of 0.11 and 0.03 μM, respectively, while two other new compounds 2, 3 showed no apparent activity.

Table 2

| Compound | HL60 (IC50, μM) | NB4 (IC50, μM) |
|----------|----------------|----------------|
| 1        | 0.11           | 0.03           |
| 2        | >100           | >100           |
| 3        | >100           | >100           |

3. Discussion

The biosynthetic pathway of puromycin has been extensively studied [21–25]. Its biosynthesis starts from the conversion of ATP. Through the sequentially catalysis of Pur10, Pur4, Pur7 and Pur3, ATP was converted to the pivot intermediate 3'-amino-3'-da. 3'-amino-3'-da was further accepted by Pur6 and condensed with the carbonyl group of tyrosine to form the amide intermediate. Following with post-modification by tailoring enzymes (Pac, Pur5, DmpM, NapH), this intermediate was finally converted into puromycin A. Surprisingly, from the biosynthetic pathway, the puromycin biosynthetic machinery indeed doesn’t have any acyl carrier protein. Thus the production of compounds 1–3 must be activated through a different mechanism from oviomycin [15].

Except for CP-involved products, phosphopantetheinylation is also essential for the biosynthesis of lysine and tetrahydrofolicate [26,27]. These central primary molecules including HSCoA play very important roles in metabolite biosynthesis, and their variation will influence many secondary metabolite biosynthesis pathways. More so, the variation of FA, PK and NRP which are used as components of the cell membrane, signal transmitters (e.g., quorum sensor) or nutrients scavengers (e.g., siderophores) can also cause physiological change and metabolic flux variation to stimulate other secondary metabolites biosynthesis [16,28]. We observed NRRL B-1832-PPtase has a slightly slow growth comparing to its wild-type strain (data not shown). This indicates the activation of non-carrier protein involving pathways confirmed that the PPtase-based approach indeed have a much broader effective scope than previously assumed. These results not only broaden the utility of this approach, but also suggest a fundamental role of the PPtase in metabolites biosynthesis regulation, which will benefit future natural products discovery and biosynthetic studies.

Acknowledgments

This work was financially supported by NSFC (Nos. 313220202, 31500049 and 31270119, 81760633) and the State Key Laboratory of Microbial Metabolism (MMLKF17-08).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.synbio.2018.02.001.

References

[1] Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 2016;79:629–61.
[2] Rutledge PJ, Challis GL. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. Nat Rev Microbiol 2013;13:509–23.
[3] He X, Liu M, Huang P, Abdel-Mageed WM, Han J, Watrous JD, Zhang J. Discovery of tanshinione derivatives with anti-MRSA activity via targeted bio-transformation. Syn Syst Biotechnol 2016;1:187–94.
[4] Zerkly M, Challis GL. Strategies for the discovery of new natural products by genome mining. Chem Commun 2014;50:9651–4.
[5] Nett M, Ikekda H, Moore BS. Genomic basis for natural product biosynthetic diversity in the actinomycetes. Nat Prod Rep 2009;26:1362–86.
[6] Qu X, Lei C, Liu W. Transcriptome mining of active biosynthetic pathways and their associated products in Streptomyces flavescens. Angew Chem Int Ed 2010;49:10561–4.
[7] Bok JW, Chiang YM, Szewczyk E, Reyes-Dominguez Y, Davidson AD, Sanchez JF, Lo HE, Watanabe K, Strauss J, Oakley BR, Wang CCC, Keller NP. Chromatin level regulation of biosynthetic gene clusters. Nat Chem Biol 2009;5:462–4.
[8] Williams RB, Henrikson JC, Hoover AR, Lee AE, Cichewicz RH. Epigenetic remodeling of the fungal secondary metabolome. Org Biomol Chem 2008;6:1895–7.
[9] Bergmann S, Schumann J, Scherlach K, Lange C, Brakhage AA, Hertweck C. Genomics-driven discovery of PKS NRPS hybrid metabolites from Aspergillus nidulans. Nat Chem Biol 2007;3:213–7.
[10] Hosaka T, Onishi-Kameyama M, Muramatsu H, Murakami K, Tsunumi Y, Kodani S, Yoshida M, Fujie A, Ochi K. Antibacterial discovery in actinomycetes strains with mutations in RNA polymerase or ribosomal protein S12. Nat Biotechnol 2009;27:462–4.
[11] Kalan L, Gessner A, Thaler CN, Wagelchen N, Zhu X, Szawiola A, Reblachtold A, Wright GD, Zechel DL. A cryptic polyene biosynthetic gene cluster in Streptomyces calvus is expressed upon complementation with a functional bldA Gene. Chem Biol 2013;20:1214–24.
[12] Schroeder V, Scherlach K, Nitzmann HW, Shelest E, Schmidt-Heck W, Schuenemann J, Martin K, Hertweck C, Brakhage AA. Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. Proc Natl Acad Sci USA 2009;106:14558–63.
[13] Seyedayamdost MR. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. Proc Natl Acad Sci USA 2014;111:7266–71.
[14] Yoon V, Nodwell JR. Activating secondary metabolism with stress and chemicals. J Ind Microbiol Biotechnol 2014;41:415–24.
[15] Zhang R, Tian W, Wang S, Yan X, Jia X, Pierens G, Chen W, Ma H, Deng Z, Qu X. Activation of natural products biosynthetic pathways via a protein modification level regulation. ACS Chem Biol 2017;12:1732–6.
[16] Beld J, Sonnenschein EC, Vickery CR, Noel JP, Burkart MD. The phosphopantetheinyl transferases: catalysis of a post-translational modification crucial for life. Nat Prod Rep 2014;31:61–108.
[17] Robins MJ, Miles RW, Samano MC, Kaspar RL. Syntheses of puromycin from the 7-aza/deaza Pair1. J Org Chem 2001;66:8204–10.
[18] Porter JN, Hewitt RI, Hesseltine CW, Krupka G, Lowery JA, Wallace WS, Wright GD, Zechel DL. A cryptic polyene biosynthetic gene cluster in Streptomyces calvus is expressed upon complementation with a functional bldA Gene. Chem Biol 2013;20:1214–24.
[19] Sergewat T, Scherlach K, Nitzmann HW, Shelest E, Schmidt-Heck W, Schuenemann J, Martin K, Hertweck C, Brakhage AA. Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. Proc Natl Acad Sci USA 2009;106:14558–63.
[20] Seyedayamdost MR. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. Proc Natl Acad Sci USA 2014;111:7266–71.
[21] Yoon V, Nodwell JR. Activating secondary metabolism with stress and chemicals. J Ind Microbiol Biotechnol 2014;41:415–24.
[22] Zhang R, Tian W, Wang S, Yan X, Jia X, Pierens G, Chen W, Ma H, Deng Z, Qu X. Activation of natural products biosynthetic pathways via a protein modification level regulation. ACS Chem Biol 2017;12:1732–6.
[23] Beld J, Sonnenschein EC, Vickery CR, Noel JP, Burkart MD. The phosphopantetheinyl transferases: catalysis of a post-translational modification crucial for life. Nat Prod Rep 2014;31:61–108.
[24] Robins MJ, Miles RW, Samano MC, Kaspar RL. Syntheses of puromycin from the 7-aza/deaza Pair1. J Org Chem 2001;66:8204–10.
[25] Porter JN, Hewitt RI, Hesseltine CW, Krupka G, Lowery JA, Wallace WS, Wright GD, Zechel DL. A cryptic polyene biosynthetic gene cluster in Streptomyces calvus is expressed upon complementation with a functional bldA Gene. Chem Biol 2013;20:1214–24.
[26] Sergewat T, Scherlach K, Nitzmann HW, Shelest E, Schmidt-Heck W, Schuenemann J, Martin K, Hertweck C, Brakhage AA. Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. Proc Natl Acad Sci USA 2009;106:14558–63.
[27] Seyedayamdost MR. High-throughput platform for the discovery of elicitors of silent bacterial gene clusters. Proc Natl Acad Sci USA 2014;111:7266–71.
[28] Yoon V, Nodwell JR. Activating secondary metabolism with stress and chemicals. J Ind Microbiol Biotechnol 2014;41:415–24.
alboniger enzymatic methylation of O-demethylpuromycin. J Biol Chem 1969;244:112–8.

[22] Pattabiraman T, Pogell BM. Biosynthesis of puromycin in Streptomyces alboniger. Possible precursors of the antibiotic in a commercial sample. Biochim Biophys Acta 1969;182:245–7.

[23] Vara J, Perez-Gonzalez JA, Jimenez A. Biosynthesis of puromycin by Streptomyces alboniger: characterization of puromycin N-acetyltransferase. Biochemistry 1985;24:8074–81.

[24] Lacalle RA, Tercero JA, Vara J, Jimenez A. Identification of the gene encoding an N-acetylpuromycin N-acetylhydrolase in the puromycin biosynthetic gene cluster from Streptomyces alboniger. J Bacteriol 1993;175:7474–8.

[25] Rubio MA, Espinosa JC, Tercero JA, Jiménez A. The Pur10 protein encoded in the gene cluster for puromycin biosynthesis of Streptomyces alboniger is an NAD-dependent ATP dehydrogenase. FEBS Lett 1998;437:197–200.

[26] Strickland KC, Hoeferlin LA, Oleinik NV, Krupenko NI, Krupenko SA. Acyl Carrier protein-specific 4′-phosphopantetheinyl transferase activates 10-formyltetrahydrofolate dehydrogenase. J Biol Chem 2010;285:1627–33.

[27] Ehmann DE, Gehring AM, Walsh CT. Lysine biosynthesis in Saccharomyces cerevisiae: mechanism of L-aminoadipate reductase (Lys2) involves post-translational phosphopantetheinylation by Lys5. Biochemistry 1999;38:6171–7.

[28] Pollakde AV, Mantri SS, Patwekar UJ, Jangid K. Quorum sensing: an under-explored phenomenon in the phylum Actinobacteria. Front Microbiol 2016;7:131.