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Evaluation of Cell Breakage Methods for Investigation of Aspects of Mycobacterial Cell Wall Biosynthesis

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Abstract: Two methods of breaking *Mycobacterium smegmatis* mc^2155 exploiting different biophysical principles were evaluated for obtaining the fractions used to monitor *in vitro* galactan build up and decaprenylphosphoryl arabinose biosynthesis. Our data suggest that cell lysis performed with the cell disrupter at 80 MPa is superior to sonication, which was used until now, especially to examine galactan polymerization.

Keywords: Mycobacteria, Cell wall, Fractionation

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1. Introduction

Mycobacterium tuberculosis, the causal agent of one of the most widespread diseases in the world is an extremely difficult pathogen for therapeutic intervention. One of the reasons is a specific structure of its cell envelope [1]. It is composed of the cell membrane, above which a covalently linked complex of peptidoglycan and arabinogalactan serves as a scaffold for the attachment of numerous mycolic acids forming the basis for the additional, highly impermeable membrane layer. Biosynthesis of mycolyl-arabinogalactan complex is a proved target of the efficient antituberculosis drugs, such as isoniazid inhibiting the NADH-dependent enoyl-[acyl-carrier-protein] reductase InhA and ethambutol acting through inhibition of arabinosyltransferases EmbA-C [2]. Just recently, DprE1 protein, part of the heteromeric epimerase enzyme DprE1/DprE2 involved in the production of precursors for arabinan polymerization, was shown to be a target of a novel drug - benzothiazinone [3]. Obviously, particular stages leading to build-up of mature mycobacterial cell envelope components may involve additional vulnerable steps that could be exploited for the development of new drugs against tuberculosis. Possibilities for identification of the genes participating in cell wall biogenesis were greatly expanded after publication of the genome sequence of *M. tuberculosis* H37Rv, which opened new opportunities for studying the physiology of this pathogen [4]. Bioinformatic examination of its genome revealed the presence of possible arabinogalactan biosynthetic cluster ranging from the gene *Rv3779* to the gene *Rv3809c* [5], and the research in the following years confirmed correctness of this prediction, as the functions of most of the genes in the cluster were identified

and, indeed, associated with the cell envelope biogenesis (for a review see [2]). We have contributed to these efforts by genetic and biochemical characterization of the galactosyltransferases Rv3782 (GlfT1) and Rv3808c (GlfT2) [6], and identification of the epimerase Rv3790/Rv3791 (DprE1/DprE2), which converts decaprenylphosphoryl ribose to decaprenylphosphoryl arabinose (DPA), thus providing the substrate for the arabinosyltransferases [7]. Crucial experimental methods for such studies are the disintegration and fractionation of the cells used in the experiments. Standard procedure for breaking the cells, which was applied in our work, was sonication. It generates alternating high-pressure and low- pressure waves in the exposed liquid. During the low-pressure cycle, the ultrasonic waves create small vacuum bubbles in the liquid that collapse violently during a high-pressure cycle. This phenomenon is termed cavitation. The implosion of the cavitation bubble causes strong hydrodynamic shear-forces. The shear forces disintegrate fibrous material into fine particles and cell wall material is being broken into small debris [8]. In the present study we evaluated an alternative breakage method using the cell disrupter for preparation of the enzymatic fractions for investigation of galactan polymerization and for biosynthesis of DPA. The method is based on the use of high pressure to force a sample through a small fixed orifice at high speed under controlled conditions. As the high-pressure piston descends, sample is introduced into the high-pressure cylinder. The piston then forces the sample through the jet at high speed. The rapid transfer of the sample from a region of high pressure to one of low pressure causes cell disruption [9].

2. Experimental

2.1. Fractionation of mycobacteria

Non-pathogenic strain *M. smegmatis* mc^2155, which has a similar cell wall structure as its pathogenic counterpart, *M. tuberculosis*, was used in the described experiments. The bacteria were grown in LB broth containing 0.05% Tween 80 (Sigma). 3 g of cells were suspended in about 6 ml of 50 mM MOPS buffer, pH 7.9, containing 5 mM 2-mercaptoethanol and 10 mM $MgCl_2$ (buffer A) at 4 C and subjected to breaking by two different means. Sonication (Soniprep 150; MSE Ltd., United Kingdom; 1-cm probe) was applied in the total time of 10 min performed in 10 cycles of 60 s pulses with 90 s cooling intervals. Alternatively, mycobacteria were disintegrated by two passages of the cell suspension in One Shot Cell Disrupter (Constant System, Ltd, United Kingdom) at 80 MPa, 200 MPa or 270 MPa, respectively. The cell lysates were centrifuged at 15,600 g for 20 min at 4 °C. The pellets were resuspended in buffer A, and Percoll (GE Healthcare) was added to achieve a 60% suspension, which was centrifuged at 15,600 g for 60 min at 4 °C. The white upper fluffy layer was collected, and Percoll was removed by repeated suspension in buffer A and centrifugation. The cell envelope fraction (P60) was resuspended in buffer A (1.5 ml per 5 g of the initial wet weight) to achieve protein concentration of 8 10 mg/ml. Membranes were obtained by centrifugation of the 15,600 g supernatant at 165,000 g for 1 hr at 4 C and suspended in buffer A (150 l per 5 g of the initial wet weight) to give a protein concentration of 25 35 mg/ml. All centrifugations were performed in ultracentrifuge OPTIMA MAX-XP (Beckman) equipped with the rotor TLA110.

2.2. Reaction mixtures and fractionation of reaction products

For monitoring of galactan biosynthesis the reaction mixtures contained 150 g of membrane proteins and/or 150 g of cell envelope proteins, 62.5 μM ATP, 2.5 mM NADH, 200 μM UDP-N-acetylglucosamine, TDP-rhamnose (prepared as described [10]), 0.25 Ci UDP-[U-^{14}C]Gal*p* (specific activity 285 mCi/mmol, GE Healthcare) and buffer A in the final volume of 80 l. Production of arabinan precursor DPA was followed in the reaction mixtures composed of 150 g of membrane protein or 150 g of cell envelope protein, 62.5 μM ATP, 50 μM NADH, 0.055 Ci phosho-[^{14}C]ribose pyrophosphate (P[^{14}C]RPP) [prepared enzymatically from [^{14}C]-glucose (specific activity 287 mCi/mmol; GE Healthcare) as described [11]] and buffer A in the final volume of 80 l. After incubation of the reaction mixtures for 1 h at 37 °C, the reactions were stopped by addition of $CHCl_3/CH_3OH$ (2 : 1; 1.5 ml). For extractions of the radiolabelled glycolipids the mixture was left rocking at room temperature for 20 min and centrifuged (3000 g). The $CHCl_3/CH_3OH$ phase was removed from the pellet and reaction products were separated by biphasic Folch wash, as described [12]. The bottom phase was dried under a stream of N_2 at room temperature, redissolved in 50 μl of $CHCl_3/CH_3OH/H_2O/NH_4OH$ (65 : 25 : 3.6 : 0.5) and quantified by the scintillation counting. Thin layer chromatography (TLC) of $CHCl_3/CH_3OH$ (2:1) extract was performed on Silica Gel plates (Merck) in $CHCl_3/CH_3OH/NH_4OH$/1 M ammonium acetate /H_2O (180 : 140 : 9 : 9 : 23) and the radiolabeled lipid bands were visualized by autoradiography. In order to obtain polymerized galactan precursors the pellet was initially washed with 0.5 ml each of 50% CH_3OH in H_2O containing 0.9% NaCl, 50% CH_3OH in H_2O and 100% CH_3OH for removal of the residual radiolabel. [^{14}C]Gal-labeled lipid-linked polymer was extracted from the pellet with 0.5 ml $CHCl_3/CH_3OH/H_2O$ (10 : 10 : 3) [13] and with 0.5 ml "E-soak" (water/ethanol/diethyl ether/pyridine/concentrated ammonium hydroxide; 15 : 15 : 5 : 1 : 0.017) [14] and quantified by the scintillation counting.

2.3. NADH oxidase assay

For measuring of NADH oxidase activity the reaction mixtures contained 50 μg of membrane proteins, or 10 μg of P60 proteins, 0.5 mM NADH and buffer A in the final volume of 200 μl. Kinetics of the reaction was measured at room temperature in 96-well plates during 15 min in the microplate reader BioTek EL808 (BioTek, United States).

3. Results and discussion

Appropriate disruption of bacteria is fundamental for preparation of the subcellular fractions to be used for monitoring of enzymatic activities. Methods employed for disintegration of the cells may affect not only the overall activities of the studied enzymes or their compartmentalization within the gained enzymatic fractions, but, especially in case of preparation of membrane fractions, also the sidedness of the obtained vesicles resulting in specific distribution of the membrane-associated proteins inside or outside the vesicles [15]. The most extensive comparison of the breaking methods exploiting various biophysical principles applied on mycobacteria was described by Rezwan et al. [16]. The authors compared cell lysis of *M. smegmatis* using cell disrupter, French press, bead beater, bead beater with lysozyme, sonication, sonication with lysozyme and lysozyme alone. The cell

lysis was followed by centrifugation at 27,000 g and the supernatant was further centrifuged at 100,000 g. Based on the analysis of the defined marker molecules in the different subfractions, the authors concluded that the 27,000 g pellet contained mostly the cell wall, while 100,000 g pellet represents the cell membrane fraction. Interestingly, with the exception of high-pressure methods (cell disrupter or French press), the cell wall fraction was highly contaminated by the membranes, as confirmed by NADH oxidase assay [16]. The NADH oxidase activity is associated particularly with the cell membrane and thus its occurrence in the cell wall fraction suggests the cross-contamination. In our initial experiment with the cell disrupter, we applied the conditions for breakage recommended by the manufacturer, i.e. 270 MPa. In this case both galactan polymerization and production of decaprenylphosphoryl arabinose were hardly detectable in the membrane and/or P60 fractions (data not shown). We thus reduced the applied pressure in the following experiments to 200 MPa and 80 MPa, in accordance with conditions described before [16 17]. We found out that effective biosynthesis of decaprenylphosphoryl arabinose took place in the enzymatic fractions obtained with disintegration in the cell disrupter at 80 MPa and it was comparable to the fractions derived from the sonicated cells. Disruption of mycobacteria at 200 MPa reduced the DPA production by approximately 50% (Fig. 1). In case of galactan biosynthesis, there were significant differences between the results obtained with enzymes from the cells lysed by sonication or by the cell disrupter. The highest quantities of the radiolabelled products were obtained with the fractions from bacteria lysed with the cell disrupter at 80 MPa (Fig. 2). Particularly, in the reaction mixture containing both membranes and P60, total incorporation of radioactive label into the lipid-linked galactan intermediates exceeded that achieved in the sonicated samples 2.5 times. Cell breakage performed at 200 MPa resulted in 54 % reduction of build up of these intermediates. In all tested P60 fractions the presence of NADH activity confirms that the cell wall-containing P60 fraction is enriched with membrane proteins, thus forming a true “cell envelope” fraction. Morita et al. [18] observed a similar fractionation pattern in mycobacteria, since the cell wall component of their fractionation scheme always contained fragments of plasma membrane. Our data suggest that the relatively gentle breaking with the cell disrupter at 80 MPa results in fractions that are more efficient in the *in vitro* polymerization of galactan, implying the need for the interaction of the components comprising the cell envelope (i. e. cell wall and cell membrane), which could be retained at such conditions. In conclusion, the cell disrupter is the suitable means for lysing mycobacteria to obtain enzymatic fractions used for investigation of aspects of mycobacterial cell wall biogenesis. Moreover, reduced time required for breakage in comparison with sonication enables handling of a larger number of samples, which is especially important in the course of identification of the enzyme functions, when various strains including the wild type, mutants and overproducers are analyzed in parallel. Another advantage is a small volume of the samples, although this can only be exploited in case of availability of the ultracentrifuge with rotors allowing fractionation of small volumes; this was the case in all of the experiments described above.

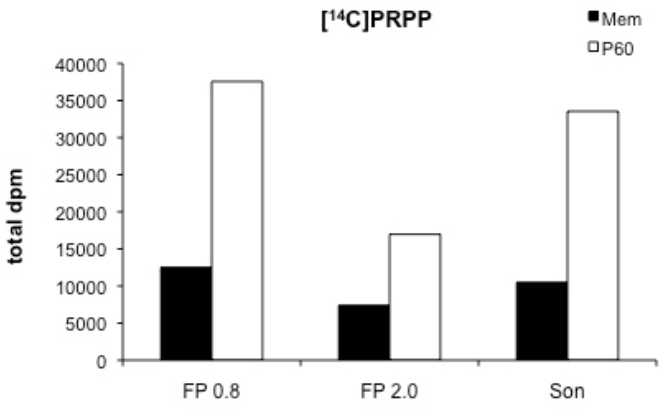

Fig. 1. Comparison of incorporation of radioactivity from [^{14}C]PRPP into organic extracts in the reaction mixtures containing equal amounts of membrane (mem) or P60 proteins, which were prepared by different means (FP 0.8 cell disrupter at 80 MPa; FP 2.0 cell disrupter at 200 MPa; Son sonication).

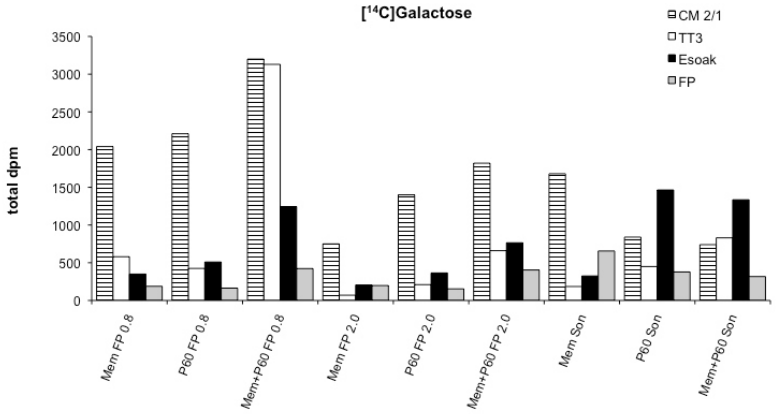

Fig. 2. Quantitative analysis of the radiolabelled galactan intermediates extracted from reaction mixtures containing equal amounts of membrane (mem) and/or P60 proteins, which were prepared by different means (FP 0.8 cell disrupter at 80 MPa; FP 2.0 cell disrupter at 200 MPa; Son sonication). CM2/1 $CHCl_3/CH_3OH$ (2:1), TT3 $CHCl_3/CH_3OH/H_2O$ (10 : 10 : 3), Esoak water/ethanol/diethyl ether/pyridine/concentrated ammonium hydroxide; 15 : 15 : 5 : 1 : 0.017), FP final pellet.

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References

[1] M. R. McNeil, P. J. Brennan: Res Microbiol, **142** (1991) 451 463.
[2] C. E. Barry, D. C. Crick, M. R. McNeil: Infect Disord Drug Targets, **7** (2007) 182 202.
[3] V. Makarov, G. Manina, K. Mikušová, U. Mollmann, O. Ryabova, B. Saint-Joanis, N. Dhar, M. R. Pasca, S. Buroni, A. P. Lucarelli, A. Milano, E. De Rossi, M. Beláňová, A. Bobovská, P. Dianišková, J. Korduláková, C. Sala, E. Fullam, P. Schneider, J. D. McKinney, P. Brodin, T. Christophe, S. Waddell, P. Butcher, J. Albrethsen, I. Rosenkrands, R. Brosch, V. Nandi, S. Bharath, S. Gaonkar, R. K. Shandil, V. Balasubramanian, T. Balganesh, S. Tyagi, J. Grosset, G. Riccardi, S. T. Cole: Science, **324** (2009) 801 804.
[4] S. T. Cole, 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, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, B. G. Barrell: Nature, **393** (1998) 537 544.
[5] A. E. Belanger, J. M. Inamine: Genetics of cell wall biosythesis. In Molecular Genetics of Mycobacteria. Ed. G. F. Hatfull and W. R. Jacobs Jr., ASM Press, Washington, D.C., 2000.
[6] M. Beláňová, P. Dianišková, P. J. Brennan, G. C. Completo, N. L. Rose, T. L. Lowary, K. Mikušová: J Bacteriol, **190** (2008) 1141 1145.
[7] K. Mikušová, H. Huang, T. Yagi, M. Holsters, D. Vereecke, W. D'Haeze, M. S. Scherman, P. J. Brennan, M. R. McNeil, D. C. Crick: J. Bacteriol. **187** (2005) 8020 8025.
[8] K.S. Suslick: Kirk-Othmer encyclopedia of chemical technology. 4th ed, J. Wiley & Sons, New York, **26** (1998) 517 541.
[9] Constant systems LTD. Cell disruption solutions. Available from: www.constantsystems.com.
[10] K. Mikušová, M. Beláňová, J. Korduláková, K. Honda, M. R. McNeil, S. Mahapatra, D. C. Crick, P. J. Brennan: J Bacteriol, **188** (2006) 6592 6598.
[11] M. S. Scherman, L. Kalbe-Bournonville, D. Bush, Y. Xin, L. Deng, M. McNeil: J Biol. Chem, **271** (1996) 29652 29658.
[12] J. Folch, M. Lees, G. H. Sloane Stanley: J. Biol. Chem, **226** (1957) 497 509.
[13] J. S. Rush, J. G. Shelling, N. S. Zingg, P. H. Ray, C. J. Waechter: J. Biol. Chem. **268** (1993) 13110 13117.
[14] W. W. Angus, R. L. Lester: Arch. Biochem. Biophys, **151** (1972) 483 495.
[15] M. Futai: J. Membrane Biol. **15** (1974) 15 28.
[16] M. Rezwan, M. A. Laneelle, P. Sander, M. Daffe: J Microbiol Methods, **68** (2007) 32 39.
[17] J. Vaubourgeix, F. Bardou, F. Boissier, S. Julien, P. Constant, O. Ploux, M. Daffe, A. Quemard, L. Mourey: J. Biol. Chem. **284** (2009) 19321 19330.
[18] Y. S. Morita, R. Velasquez, E. Taig, R. F. Waller, J. H. Patterson, D. Tull, S. J. Williams, H. Billman-Jacobe, M. J. McConville: J. Biol. Chem, **280** (2005) 21645 21652.