The Journal of Antibiotics (2009) 62, 315–318

www.nature.com/ja

ORIGINAL ARTICLE

A cell-based screening system for detection of inhibitors toward mycobacterial cell wall core

Peng Gao, Yan Guan, Danqing Song and Chunling Xiao

Mycobacterium tuberculosis **and nonpathogenic bacteria,** ***Corynebacterium glutamicum,*** **possess a common and unusual cell wall architecture. A cell-based screening system was designed to identify novel compounds interacting with the synthesis, assembly or regulation of the** ***M. tuberculosis*** **cell wall.** ***C. glutamicum*** **was tested in a paired medium assay in 96-well plates with natural product extracts and pure chemical compounds in the presence and absence of the osmotic stabilizer, sorbitol and some ions. Growth was visually examined over a 12-h period and detected with a microplate reader for absorbance at 544 nm. Screening hits from the osmotic stabilizer rescue were then examined by mycolic acid analysis to confirm the effect on cell wall integrity.**
The Journal of Antibiotics (2009) **62,** 315–318; doi:10.1038/ja.2009.34; published online 15 May 2009

Keywords: cell wall core; *Corynebacterium glutamicum*; *Mycobacterium tuberculosis*; mycolic acid; screening system

INTRODUCTION

The cell wall is crucial for the survival of *Mycobacterium tuberculosis*. Enzymes related to the process of biosynthesis, assembly and regulation of cell wall are particularly good molecular targets, because there are no homologs in the mammalian system.[1] *M. tuberculosis* and nonpathogenic bacteria, *Corynebacterium glutamicum,* share a common and unusual cell wall architecture.[2] The cell wall core consists of a peptidoglycan layer and a lipid mycolic acid layer that are connected by the polysaccharide, arabinogalactan.[1] The *C. glutamicum* is a more convenient and representative test strain than are mycobacteria because of its short growth cycle, its nonpathogenicity and similar cell wall architecture to *M. tuberculosis*. *M. smegmatis* has been preferred in the past as the screening organism, as it is a species related to *M. tuberculosis*. However, the mutant of key cell wall biosynthetic enzymes cannot be conducted and therefore this organism is not a reliable screening organism.[2–5]

Inhibition of the key enzymes involved in the synthesis, assembly or regulation of the *M. tuberculosis* cell wall results in cell disruption and death of *M. tuberculosis* and *M. smegmatis*. However, in the case of the *C. glutamicum* inhibition of cell wall, biosynthetic enzymes result in slow growth and not in cell death.[2] The addition of an osmotic stabilizer can protect the cell from cell wall disruption.[2,6] Arabinofuranosyltransferase, coded by *AftB*, is involved in the terminal step of cell wall arabinan biosynthesis, which catalyzes the linkage of the last arabinose to arabinogalactan. In the mutant strain with the *aftB* mutant gene, mycolic acid cannot link with arabinogalactan and the mutant grows slower than does the wild type. The addition of sorbitol almost completely restores the growth of this mutant.[5]

To confirm the interaction between screening hits and cell wall targets and to differentiate these effects from other possibilities related to rescue from osmotic pressure lysis, we developed a simple method to analyze the change of cell wall. Although electron microscopy can show morphological changes better than the optical microscope, the electron micrograph is not compatible with high-throughput screening (HTS).[7] As mycolic acid is the outer layer of the cell wall,[5] mycolic acid analysis by TLC could show the change in cell wall and be conducted by HTS. In this report, we have successfully established an HTS system, using *C. glutamicum* as the test strain. Two of the inhibitors identified through screening were confirmed by mycolic acid analysis.

MATERIALS AND METHODS

Bacterial strains, growth conditions and antibiotics

Corynebacterium glutamicum ATCC 13032 (the wild-type strain, referred to simply as *C. glutamicum*) was purchased from the China Center of Industrial Culture Collection (CICC, Beijing, China), and was cultured in BHI (3.7% brain–heart infusion (Difco, Sparks, MD, USA)) and BHIS (3.7% brain–heart infusion, 10.92% sorbitol, 1% NaCl and 0.05% $MgSO_4$) at 30 °C. The mutant strain, *C. glutamicum* Δ*aftB*, was provided by Dr L Eggeling.[5] *M. smegmatis mc²155* was purchased from the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), and was grown in medium no. 54 (1% tryptone, 0.5% malt extract, 0.5% yeast extract, 0.5% casein acid hydrolysate (Sigma, St Louis, MO, USA), 0.2% beef extract, 0.2% glycerol, 0.005% Tween 80 and 0.1% $MgSO_4$ $7H_2O$, pH 7.2) at 37 °C. Solid growth medium was prepared by the addition of 1.5% agar to the liquid medium.

The antibiotics used in this study are cephalothin, ethambutol (EMB), isoniazid, vancomycin, streptomycin, levofloxacin, rifampicin, actinomycin D,

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, China
Correspondence: Professor CL Xiao, Key Lab, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Peking Union Medical College, Tiantanxili 1, Chongwen District, Beijing 100050, China.
E-mail: xiaoimb@gmail.com
Received 6 January 2009; revised 14 April 2009; accepted 17 April 2009; published online 15 May 2009

lincomycin, tetracycline and D-cycloserine. All of them were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and dissolved in sterile water or DMSO, sterilized by filtration and diluted with sterile water.

Preparation of cells

Bacterial stock cultures were streaked on BHI agar plates and incubated overnight at 30 °C. A single colony from the plate was used to inoculate BHI, and was incubated for 12 h with shaking at 200 r.p.m. at 30 °C. When the cell density reached 4.5–5.5 OD at 600 nm, the culture was diluted to 3 OD at 600 nm and was stored at 4 °C as seed culture for screening. The growth curve and the inhibition of EMB-treated control were measured to obtain the optimal inoculum concentration and culture period.

Screening protocol

The screen was developed as a 96-well plate test. Except for the blank control, 180 µl of *C. glutamicum* culture (the medium with 0.3% inoculum from the seed culture) and 20 µl of test samples or controls to the final volume 200 µl were added to each well. Each plate contained 80 wells as the sample group, with 1 µg ml^{-1} of test compounds, and the remaining 16 wells were the control group, which included four wells with 1‰ DMSO (regular growth control), four wells with 180 µl BHI/BHIS medium and 1‰ DMSO (blank control), four wells with 1 µg ml^{-1} rifampicin (negative control) and four wells with 1 µg ml^{-1} EMB (positive control). The OD at 544 nm was measured after the plates were incubated at 12 h at 30 °C. The inhibition rate can be calculated as follows: Inhibition (%)=(OD of regular growth control−OD of sample)/(OD of regular growth control−OD of blank control)×100%.

The inhibition difference (Δinhibition) between the BHI medium and the BHIS medium was calculated as the screening factor.

$$\Delta\text{inhibition} = I_{\text{BHI}} - I_{\text{BHIS}}$$

I_{BHI}, the inhibition of samples/controls in BHI medium; I_{BHIS}, the inhibition of samples/controls in BHIS medium.

In the screening system, 1 µg ml^{-1} EMB and rifampicin were used as controls to monitor the reproducibility of the screen. When the Δinhibition of the EMB-treated sample was larger than 50% and the Δinhibition of rifampicin was smaller than 5%, the results were considered to be reliable.

Application of the screening system to known antibiotics and HTS assay

Different antibiotics with diverse targets were tested against *C. glutamicum* according to the screening protocol. The final concentration of known antibiotics and samples was 1 µg ml^{-1}. The screen was validated using the Δinhibition of positive and negative antibiotics with a known mode of action.

After screening the compound library and natural product extracts, the positive samples were added to the culture at the appropriate concentration to characterize the mycolic acid in methanol extracts of lysates.

The mycolic acid analysis

Corynebacterium glutamicum were harvested at an absorbance of 10–15 OD at 600 nm. The cultivation of *C. glutamicum* Δ*aftB* and wild type treated with positive samples required two precultures. First, a 5-ml BHIS culture was grown for 8 h, which was then inoculated with 50 ml BHIS and cultured for 15 h. It was then used to inoculate a 100 ml BHIS culture and grown to an absorbance of 1 OD at 600 nm. Each culture was harvested after reaching an absorbance of 3 OD at 600 nm.[2,5]

The cells were harvested, washed and freeze dried. The cells (100 mg) were extracted by adding 2 ml of methanol–toluene–oil of vitriol, 30:15:1 (v/v), for 18 h at 75 °C. After being cooled to room temperature, they were extracted by 2 ml petroleum ether. The supernatants were then injected into a NH_4HCO_3 column and washed with petroleum ether (2 ml), and the extract was centrifuged (3000 r.p.m., 10 min). After centrifugation, the clear supernatants were again dried and re-suspended in petroleum ether (100 µl). An aliquot (10 µl) from each strain was subjected to TLC using silica gel plates (5735 silica gel 60F_{254}; Merck, Darmstadt, Germany), developed in petroleum ether/acetone (95:5, v/v) and charred using 10% sulphuric acid in ethanol at 100 °C to reveal corynomycolic acid methyl esters.[5,8]

MIC testing

In vitro activity (MICs) was determined by inoculating 5×10^5 cells per ml of *M. smegmatis* into medium no. 54 in 96-well plates. The final volume in each well was 100 µl. The MIC was defined as the minimum concentration resulting in a cell density less than 0.01 OD at 600 nm,[9] which corresponded to no visible growth, after incubating for 48 h at 37 °C.

RESULTS

Screening conditions

Inoculum concentrations. The sensitivity of the screen is dependent on the OD of the culture at the beginning. The growth curve of *C. glutamicum* (Figure 1a) shows that inoculum concentrations lower than 1% of the seed culture are optimal for observing significant differences between the density at stationary phase and the starting culture density. It was found that 12 h is sufficient for the growth period. In the BHI medium, 0.3% seed culture presented the most significant Δinhibition of *C. glutamicum* (Figure 1b). However, in the

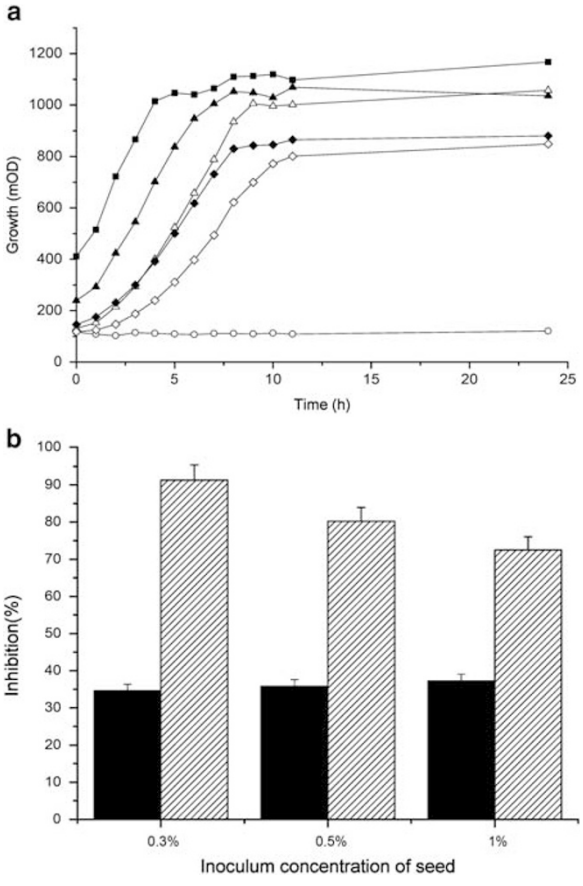

Figure 1 Growth curves of *C. glutamicum* in BHI medium and the inhibition by EMB of *C. glutamicum* grown in BHI and BHIS media, starting with different concentrations of seed inocula. The absorbance was detected with microplate reader at 544 nm. (**a**) Growth of *C. glutamicum* with different inoculum concentrations of seed: 5% (■), 1% (▲), 0.5% (△), 0.3% (◆), 0.1% (◇) and blank (○). (**b**) Effect of EMB at a concentration of 1 µg ml^{-1} on the growth of *C. glutamicum* in BHI and BHIS media started with different seed inocula concentrations. The black bar is the inhibition of *C. glutamicum* in the BHIS medium; the diagonal bar is that in the BHI medium.

Table 1 The inhibition of different target antibiotics to *Corynebacterium glutamicum* in both of the media

Antibiotics	*ΔInhibition (%)*[a]
Cephalothin	17.5 ± 2.23
EMB	56.5 ± 5.71
Isoniazid	—
Vancomycin	4.4 ± 1.33
Streptomycin	5.1 ± 1.02
Levofloxacin	4.9 ± 1.56
Rifampicin	2.0 ± 0.88
Actinomycin D	1.2 ± 0.80
Lincomycin	−0.5 ± 0.22
Tetracycline	2.7 ± 0.53
D-Cycloserine	13 ± 3.01[b]

Abbreviation: EMB, ethambutol.
[a]The Δinhibition test with compounds at 1 μg ml^{-1}.
[b]D-Cycloserine was tested at 10 μg ml^{-1}; —, without any inhibition.

BHIS medium, changing the inoculum concentration did not produce a measurable change of inhibition.

Selection of control drug concentrations: 1 μg ml^{-1} of EMB showed a large Δinhibition of 56.5 ± 5.71; higher concentrations of EMB, 10 and 100 μg ml^{-1}, yielded lower Δinhibitions of 22.8 ± 2.13 and 9 ± 0.87, respectively. These results obtained in the BHIS medium show that *C. glutamicum* cannot grow at higher EMB concentrations and no differential growth can be measured. Thus, 1 μg ml^{-1} was chosen as the concentration of compounds to be tested in the HTS assay.

Application of the screening system to known antibiotics

Using the method described in the screening protocol, we tested the inhibition of known antibiotics in *C. glutamicum*. According to the results (Table 1), two conclusions were reached. The results obtained with EMB, D-cycloserine and negative controls showed that the screening system was reliable. D-Cycloserine inhibited the growth of *C. glutamicum* at 10 μg ml^{-1}, and the inhibition (%) in the BHI medium was 55 ± 5.0. In the case of D-cycloserine, the activity against *C. glutamicum* was different from its activity against *M. tuberculosis* and *M. smegmatis* (MICs are 25 and 75 μg ml^{-1}, respectively). Streptomycin and tetracycline, which target the 30S subunit of the bacterial ribosome, and lincomycin, which targets the 50S subunit of the bacterial ribosome, showed a similar activity against *C. glutamicum* when tested in both media. Rifampicin and actinomycin D, which target the initial step of RNA synthesis and DNA replication, respectively, were negative in the screen and served as negative controls. Levofloxacin, which targets DNA gyrase subunit A, was also negative in the screen.

Some differences between *Corynebacterium* and *Mycobacterium* were noted. Cephalothin, which does not inhibit the growth of mycobacteria, was growth inhibitory in the assay, whereas isoniazid, which targets tuberculosis cell wall, did not have an effect on *Corynebacterium*.

Comparing the Δinhibition among the selected antibiotics, we chose 10% as the cutoff criteria for selecting positive samples. At the same time, the inhibition in the BHI medium showed larger than 50%.

The utilization of the screening system and further validation

The assay was used to screen a compound library with 1680 compounds. Fifteen compounds with mycobacteria inhibitory activity were found, and the hit rate was 0.9%.

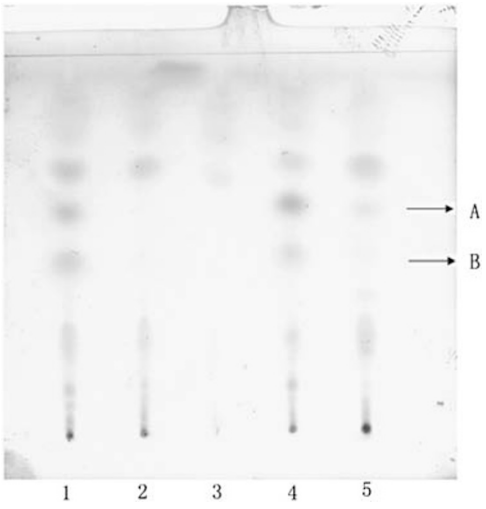

Figure 2 The TLC pattern of whole-cell mycolic acids in methanol lysates of *C. glutamicum*. The samples extracted from (1) *C. glutamicum* wild type; (2) *C. glutamicum* Δ*aftB*; (3) the control of EMB; (4) *C. glutamicum* wild type treated with compound 2008551; and (5) *C. glutamicum* wild type treated with compound 2009461. The spots: (A) α-mycolates, R_f=0.6; (B) keto-mycolates, R_f=0.45.

To confirm the interaction of the positive samples with the cell wall, we chose two of the hits and analyzed their effect on the cell wall mycolic acid content. We compared the mycolic acid in methanol extracts of lysates from wild type treated with screening hits with the mycolic acid present in the cell walls of the wild-type strain treated with EMB or the cell wall of the Δ*aftB* mutant, which is deficient of mycolic acid. EMB is known to target the assembly of arabinogalactan.[7] The cell wall of mutant and EMB-treated wild-type organisms lacks α-mycolates and/or keto-mycolates (Figure 2). A similar pattern, that is, a loss of mycolic acids, was noted in one of the hit compound (compound 2009461 but not compound 2008551)-treated cell walls, suggesting that the components of cell wall may be the target for at least one of the hits, and showing that screen could be reliable to find cell wall inhibitors. The compound, 2009461 (Figure 3a), is erythromycin A 11, 12-cyclic carbonate, which is mainly known to target protein biosynthesis, whereas our study indicates that it has an effect on cell wall biosynthesis (Figure 3b). The inhibition of *C. glutamicum* in BHI and BHIS media by EMB, rifampicin and a screening sample, 2009461, indicates that because compound 200946 has other effects, BHIS medium could not complement the growth of *C. glutamicum* treated with compound 2009461 as well, as was seen with EMB. The MICs obtained with compound 2009461 and EMB against *M. smegmatis* were 0.4 and 0.8 μg ml^{-1}, respectively. The specific enzyme targets of the hits are still being determined.

DISCUSSION

In this study, we established a screening system on the basis of differential growth rates of *C. glutamicum* in two growth media. Through this screening system, we could identify hits targeting the synthesis, assembly or regulation of cell wall core biosynthesis, which is dependent on unknown key enzymes. The screening system incorporated the ability to exclude false-positive hits that have clouded other molecular-based screening systems. Being a cell-based system, the screen has the advantage of identifying only those compounds that are active in whole cells.

Furthermore, although *C. glutamicum* and *M. tuberculosis* have a common cell wall architecture, there are differences between them.

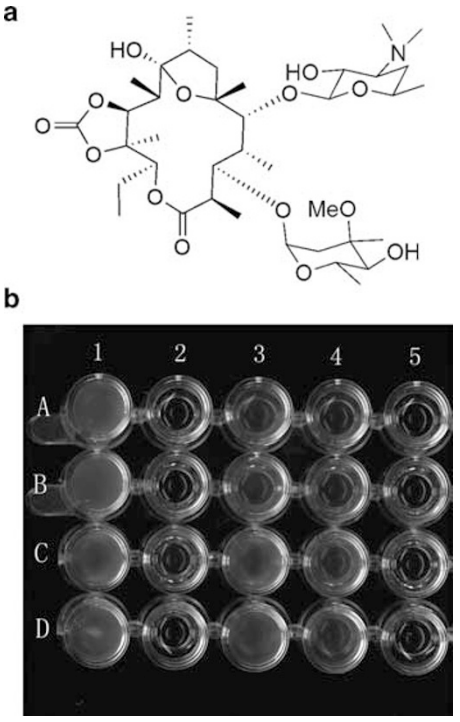

Figure 3 The structure of 2009461 and the inhibition of *C. glutamicum* by EMB, 2009461 and rifampicin grown in BHI and BHIS media. (**a**) The structure of 2009461, which is erythromycin A 11, 12-cyclic carbonate. (**b**) The compounds were all used at 1 μg ml^{-1}. Lines A and B: BHI medium; lines C and D: BHIS medium. (1) *C. glutamicum* grown with 20 μl of 1% DMSO; (2) blank medium with 20 μl of 1% DMSO; (3) growth system with 20 μl EMB; (4) growth system with 20 μl 2009461; and (5) growth system with 20 μl rifampicin.

C. glutamicum is more sensitive to antibiotics than is tuberculosis. Therefore, we needed to test the antimycobacterial activity of positive samples using *M. smegmatis* and *M. tuberculosis*. Isoniazid is a prodrug,[10] and the BLAST analysis indicated that *C. glutamicum* does not have the KatG gene, which has been proposed to activate isoniazid. Nonetheless, we choose *C. glutamicum* as the test strain because of its short growth period and the convenience for further study in mutant organisms that are available, such as *embAB*,[4] *aftA*[2] and *aftB*,[5] which are unavailable in *M. smegmatis*.

It was noted that during screening, when the Δinhibition was smaller than 10% and the inhibition was larger than 90%, the samples should be diluted to 1/10 of the former concentration and re-tested. If the results after being diluted are larger than 10%, they could be positive samples.

In addition, the compounds identified from this screening system may target any key enzymes related to the cell wall. The specific target still needed further experimentation. Compounds that target proteins related to protecting the cell from osmotic pressure may also be identified as positive. These hits were eliminated by an analysis of mycolic acid in methanol extracts. Further verification of hits will be essential using mutant construction and enzyme expression.

ACKNOWLEDGEMENTS

We thank L Eggeling for providing the mutant strain. This research was supported by National Natural Science Foundation of China (NSFC), and the project number is 30873186.

1 Barryl, C. E., Crick, D. C. & McNeil, M. R. Targeting the formation of the cell wall core of *M. tuberculosis*. *Infect. Disord. Drug Targets* **7,** 182–202 (2007).
2 Alderwick, L. J., Seidel, M., Sahm, H., Besra, G. S. & Eggeling, L. Identification of a novel Arabinofuranosyltransferase (AftA) involved in cell wall Arabinan biosynthesis in *Mycobacterium tuberculosis*. *J. Biol. Chem.* **281,** 15653–15661 (2006).
3 Berg, S., Kaur, D., Jackson, M. & Brennan, P. J. The glycosyltransferases of mycobacterium tuberculosis; roles in the synthesis of arabinogalactan, lipoarabinomannan, and other glycoconjugates. *Glycobiology* **17,** 35–56R (2007).
4 Alderwick, L. J. *et al.* Deletion of Cg-emb in corynebacterianeae leads to a novel truncated cell wall arabinogalactan, whereas inactivation of Cg-ubiA results in an arabinan-deficient mutant with a cell wall galactan core. *J. Biol. Chem.* **280,** 32362–32371 (2005).
5 Seidel, M. *et al.* Identification of a novel arabinofuranosyltransferase AftB involved in a terminal step of cell wall arabinan biosynthesis in Corynebacterianeae, such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. *J. Biol. Chem.* **282,** 14729–14740 (2007).
6 Frost, D. J., Brandt, K. D., Cugier, D. & Goldman, R. A whole-cell *Candida albicans* assay for the detection of inhibitors towards fungal cell wall synthesis and assembly. *J. Antibiot.* **48,** 306–310 (1995).
7 Radmacher, E. *et al.* Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*. *Microbiology* **151,** 1359–1368 (2005).
8 Minnikin, D. E., Minnikin, S. M., Parlett, J. H., Goodfellow, M. & Maqnusson, M. Mycolic acid patterns of some species of Mycobacterium. *Arch. Microbiol.* **259,** 446–460 (1985).
9 Sun, D., Cohen, S., Mani, N., Murphy, C. & Rothstein, D. M. A pathway-specific cell based screening system to detect bacterial cell wall inhibitors. *J. Antibiot.* **55,** 279–287 (2002).
10 Zhang, Y. & Amzel, L. M. Tuberculosis drug targets. *Curr. Drug Targets* **3,** 131–154 (2002).