The Journal of Microbiology (2012) Vol. 50, No. 2, pp. 235–240
DOI 10.1007/s12275-012-1459-0

Purification and Structure Analysis of Mycolic Acids in *Corynebacterium glutamicum*

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(Received September 15, 2011 / Accepted December 14, 2011)

Corynebacterium glutamicum **is widely used for producing amino acids. Mycolic acids, the major components in the cell wall of *C. glutamicum* might be closely related to the secretion of amino acids. In this study, mycolic acids were extracted from 5 strains of *C. glutamicum*, including ATCC 13032, ATCC 13869, ATCC 14067, L-isoleucine producing strain IWJ-1, and L-valine producing strain VWJ-1. Structures of these mycolic acids were analyzed using thin layer chromatography and electrospray ionization mass spectrometry. More than twenty molecular species of mycolic acid were observed in all 5 strains. They differ in the length (20–40 carbons) and saturation (0–3 double bonds) of their constituent fatty acids. The dominant species of mycolic acid in every strain was different, but their two hydrocarbon chains were similar in length (14–18 carbons), and the meromycolate chain usually contained double bonds. As the growth temperature of cells increased from 30°C to 34°C, the proportion of mycolic acid species containing unsaturated and shorter hydrocarbon chains increased. These results provide new information on mycolic acids in *C. glutamicum*, and could be useful for modifying the cell wall to increase the production of amino acids.**

Keywords: *Corynebacterium glutamicum*, mycolic acids, electrospray ionization mass spectrometry

Introduction

Mycolic acids are branched chain fatty acids found in CMN bacteria, which include the families *Corynebacteriaceae*, *Mycobacteriaceae*, and *Nocardiaceae*. CMN bacteria are Gram-positive bacteria characterized by their unique mycolic acid-containing cell wall structures (Brennan and Nikaido, 1995; Stackebrandt *et al.*, 1997). Mycolic acids in *Mycobacterium tuberculosis*, a human pathogen that causes tuberculosis, have been intensively studied (George *et al.*, 1995; Barry *et al.*, 1998; Glickman *et al.*, 2000; Asselineau *et al.*, 2002; Takayama *et al.*, 2005). They covalently bind to other molecules, form an outer layer of the cell wall and engender unusually low permeability, which might be responsible for the drug resistance of *M. tuberculosis* (Nikaido, 1994). Mycolic acids are also the major components in the surface bilayer of the cell wall in *Corynebacterium glutamicum* (Eggeling and Sahm, 2001).

C. glutamicum is widely used for industrial production of amino acids (Abs *et al.*, 1967; Liebl *et al.*, 1991; Amador *et al.*, 1999). In recent years, metabolic engineering in *C. glutamicum* to increase the yield of amino acids has been initiated (Xu *et al.*, 2010a, 2010b, 2011), focusing on enhancement of the amino acid synthetic pathway and the amino acid efflux system. Addition of detergents and penicillin resulted in a higher efflux of amino acids, suggesting that the cell wall plays an important role on the secretion of amino acids (Eggeling and Sahm, 2001). Since mycolic acids are the major components in the outer layer of *C. glutamicum*, they would be critical for amino acid efflux. Therefore, it is necessary to analyze the structure of mycolic acids in different strains of *C. glutamicum*, and to obtain useful information for improving the permeability of the cell envelope of *C. glutamicum* by metabolic engineering.

Available methods for characterization of mycolic acids in *Mycobacteria* are electron impact gas chromatography mass spectrometry (Yano *et al.*, 1978; Tomiyasu *et al.*, 1986; Nishiuchi *et al.*, 2000) and silver ion-containing silica thin layer chromatography (TLC) followed by mass spectrometry analysis (Alshamaony *et al.*, 1976a, 1976b). These approaches require the hydrolysis of mycolic acids from the bound polysaccharides, the conversion of mycolic acids into methyl esters, or the further conversion of the methyl esters into trimethylsilyl derivatives. They are not appropriate for isolating mycolic acids from *C. glutamicum* because 90% of them are not covalently bound (Puech *et al.*, 2001).

In this study, mycolic acids were purified from 5 different strains of *C. glutamicum* by diethyl ether extraction and preparative TLC, and then their structures were analyzed using electrospray ionization mass spectrometry (ESI/MS). The structural differences of mycolic acids were also analyzed when cells were grown at different temperatures. These results could provide new information for modifying the cell wall in *C. glutamicum* to increase the yield of amino acids during production.

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Materials and Methods

Extraction and purification of mycolic acids

Overnight cultures of *C. glutamicum* ATCC 13032, ATCC 13869, ATCC 14067, L-isoleucine producing strain IWJ-1, and L-valine producing strain VWJ-1 were inoculated into 30 ml LB medium supplemented with 5 g/L glucose at 30°C or 34°C. Cells were harvested at the late-stationary phase by centrifugation, and the pellets were dried under vacuum, and then stored at -20°C.

Approximately 50 mg of dry cells were resuspended in a mixture of 1 ml of 7 M KOH and 7 ml of 2-methoxyethanol, transferred into a glass bottle and sonicated in a water bath for 10 min at 21°C. The mixture was heated at 100°C for 1 h, cooled to room temperature, and neutralized using 2.5 ml of 20% H_2SO_4. The mixture was extracted thrice using diethyl ether; these supernatants were combined and washed thrice with distilled water. The ether layer was transferred into a glass tube, and the solvent was evaporated in a water bath at 60°C under a stream of nitrogen. The dried lipids were mainly mycolic acids, and were stored at -20°C.

Separation and purification of mycolic acids by thin-layer chromatography

Crude mycolic acids were resuspended in 0.5 ml diethyl ether, and 15 μl of the solution were spotted on a silica gel-coated plate. The plate was developed twice in a solvent of N-hexane and diethyl ether (4:1, v/v). Mycolic acids were visualized by spraying plates with 10% phosphomolybdic acid hydrate in ethanol, followed by heating at 125°C for 3 min.

To further purify mycolic acids, preparative TLC was employed. When the developed TLC plate was sprayed with distilled water, mycolic acid bands could be seen transiently as white zones. These zones were marked with a pencil and scraped off after the plate was dry. The silica chips were resuspended in diethyl ether, stirred at room temperature for 2 h, then the mixture was filtered through a 0.22 μm membrane. The solvent was evaporated in a water bath at 60°C under a stream of nitrogen. The purified mycolic acids were stored at -20°C.

Analysis of mycolic acids by electrospray ionization mass spectrometry

Mycolic acid samples were dissolved in diethyl ether and subjected to ESI/MS analysis in the negative ion mode, where the cone voltage was set at 50 V and collision energy at 6 eV. The MS/MS experiments were carried out with collision energy set at 20 eV. The mass spectra were acquired on a Waters SYNAPT Q-TOF mass spectrometer equipped with an ESI source. Data acquisition and analysis were performed using MassLynx V4.1 software.

Results

TLC and ESI/MS analysis of mycolic acids in *C. glutamicum*

Mycolic acids purified from cells of *C. glutamicum* ATCC 13032, ATCC 13869, ATCC 14067, IWJ-1, and VWJ-1 were separated on TLC. As shown in Fig. 1, only one major band was observed in all 5 samples. This band migrated at a similar rate and had similar intensity for each sample. To identify their structures, these lipids were further analyzed by ESI/MS in negative ion mode. Similar patterns were observed in the spectra of all 5 samples (Fig. 2). More than twenty major peaks were observed in each spectrum, but their relative intensities were slightly different (Table 1). The most prominent peak was at m/z 495.4 for samples from ATCC 13032, ATCC 13869, IWJ-1, and VWJ-1, and at m/z 547.5 for ATCC 14067. Other major peaks were observed at m/z values differing by 28 or 2 mass units. These m/z values are consistent with the molecular masses of mycolic acids, suggesting these peaks all derived from mycolic acids that differ by the number of methylene units or double bands. Peaks at m/z 355.3, 383.3, 411.3, 439.4, 467.4, 481.4, 495.4, 509.4, 537.4, 565.5, 579.5 could be derived from mycolic acids containing saturated hydrocarbon chains, and peaks at m/z 409.3, 437.4, 465.4, 477.4, 491.4, 493.4, 503.5, 519.5, 521.4, 545.5, 547.5, 563.5 from mycolic acids containing double bonds. Minor peaks at m/z 477.4, 491.4, 519.5, and 547.5 might come from mycolic acids containing two double bonds, and minor peaks at m/z 503.5 and 545.5 from mycolic acids containing three double bonds. The predicted numbers of the carbons and double bands in these mycolic acids are listed in Table 1. There are minor peaks showing odd carbon numbers in the molecules, such as ones at m/z 481.4, 509.4, and 537.4 for saturated mycolic acids and ones at m/z 477.4 and 563.5 for unsaturated molecules. These results suggest that there are many mycolic acid species in *C. glutamicum*, differing in the length and saturation of their hydrocarbon chains.

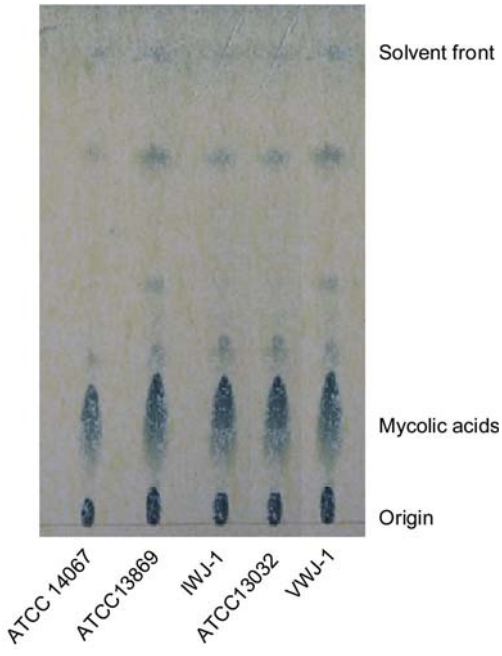

Fig. 1. **TLC analysis of mycolic acids extracted from 5 different strains of *C. glutamicum*.**

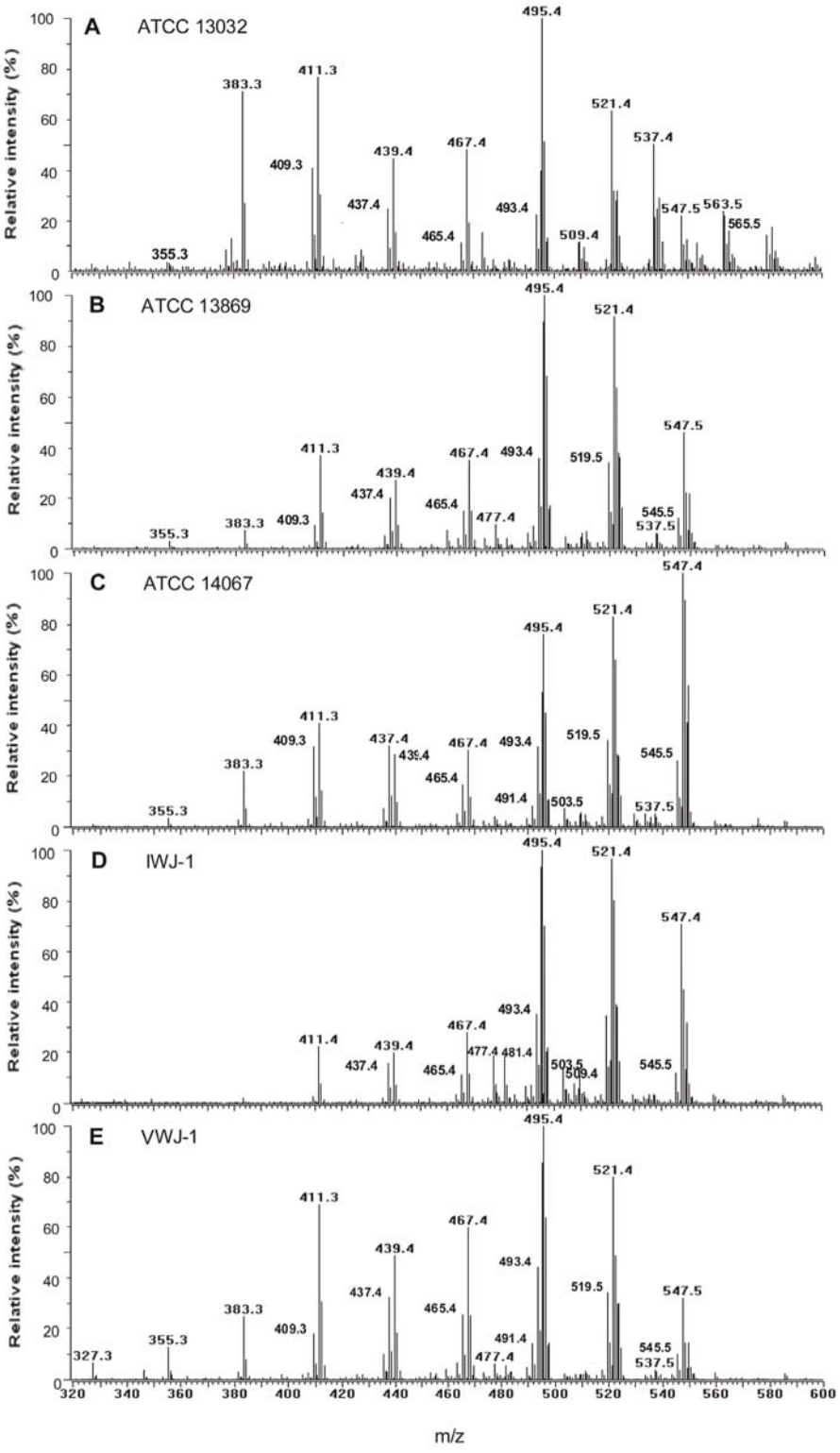

Fig. 2. **ESI/MS analysis of purified mycolic acids from 5 different strains of *C. glutamicum*.**

Structure variation of mycolic acids in *C. glutamicum* when grown at different temperatures

To study the influence of growth temperature on the structure and composition of mycolic acids, *C. glutamicum* ATCC 14067 cells were grown at both 30°C and 34°C. Mycolic acids were extracted, purified, and analyzed by ESI/MS. Compared to cells grown at 30°C, peak intensities in the spectrum of mycolic acids from cells grown at 34°C changed (Table 2). For example, the most prominent peak for mycolic acids synthesized at 30°C was at m/z 547.5, but it was at m/z 495.4 for mycolic acids synthesized at 34°C. The relative

Table 1. **Information on the structure of major mycolic acids from 5 different strains of *C. glutamicum***

m/z	Number of carbons/ double bonds	Relative intensity (%)				
		ATCC 13032	ATCC 13869	ATCC14067	IWJ-1	VWJ-1
355.3	22:0	4	3	4	<1	15
383.3	24:0	65	7	21	2	28
409.3	26:1	38	9	31	3	21
411.3	26:0	72	37	40	22	64
437.4	28:1	24	20	31	16	34
439.4	28:0	44	27	28	19	48
465.4	30:1	10	15	16	10	24
467.4	30:0	47	35	30	27	58
477.4	31:2	5	9	5	18	6
481.4	31:0	4	4	2	18	6
491.4	32:2	<1	9	8	7	12
493.4	32:1	22	36	31	33	43
495.4	32:0	100	100	76	100	100
503.5	33:3	3	5	8	13	<1
509.4	33:0	12	6	5	10	4
519.5	34:2	5	34	34	32	34
521.4	34:1	64	92	84	96	71
537.4	35:0	52	6	5	2	4
545.5	36:3	<1	12	26	10	8
547.5	36:2	23	46	100	66	29
563.5	37:1	25	1	2	<1	<1
565.5	37:0	16	1	1	<1	<1
579.5	38:0	15	<1	1	1	<1

intensities of peaks at m/z 383.3, 409.3, 411.3, 437.4, 439.4, 465.4, 467.4, 493.4 were higher for mycolic acids synthesized at 34°C than at 30°C, suggesting that at higher temperature the cell synthesizes more mycolic acids containing unsaturated and shorter hydrocarbon chains. This might be a way for bacteria to protect themselves at higher temperature, by improving the fluidity of the cell wall (Sinensky, 1974; Suutari and Laakso, 1994; Denich *et al.*, 2003).

ESI/MS/MS of the major mycolic acid species at m/z 495.5, 521.5, and 547.5

Mycolic acids consist of a meromycolate chain and an α-branch (Fig. 3A). When analyzed by MS/MS, the collision could eliminate the meroaldehyde residue from the molecular ions of mycolic acid, forming carboxylate anions containing α-alkyl chains (Fig. 3A). Therefore, the structural information from the fragment ions observed in the MS/MS spectrum could afford structural assignment of the mycolic acids, including the lengths of the meromycolate chain and the α-branch.

The molecular ions at m/z 495.5, 521.5, and 547.5 were further analyzed by high resolution MS/MS (Figs. 3B–3D). The peak at m/z 255.2 observed in all spectra has the ion mass expected for a C16:0 fatty acid, suggesting that all three molecular ions contain the C16:0 α-branch. Peaks at 227.2 and 281.2 observed in some spectra could derive from the C14:0 and C18:1 α-branches, respectively. The mycolic acid containing a C18-meromycolate chain and a C16 α-branch is indicated here as 18:0/16:0 mycolic acid. Therefore, the ions at m/z 495.5 could derive from either 18:0/14:0 or 16:0/16:0 mycolic acids, because they could contain either C14:0 or C16:0 α-branches, based on the major peaks at m/z 227.2 and 255.2 in the MS/MS spectrum (Fig. 3B). Peaks at m/z 255.2 and 281.2 were the major ones in the MS/MS spectrum of the ions at m/z 521.5, they could derive from either 18:1/16:0 or 16:0/18:1 mycolic acids (Fig. 3C). Three major peaks at m/z 227.2, 255.2, and 281.2 were observed in the MS/MS spectrum of the ions at m/z 547.5, they could derive from 22:2/14:0, 20:2/16:0 or 18:1/18:1 mycolic acids (Fig. 3D). These results indicate that the mycolic acids have isomeric structures and produce the same m/z value in the MS spectra, but that in each mycolic acid the two hydrocarbons are similar in length

Table 2. **Comparison of the structure of major mycolic acids from *C. glutamicum* ATCC 14067 grown at different temperatures**

m/z	Number of carbons and double bonds	Relative intensity (%)	
		30°C	34°C
383.3	24:0	21	57
409.3	26:1	31	51
411.3	26:0	40	64
437.4	28:1	31	32
439.4	28:0	28	39
465.4	30:1	16	18
467.4	30:0	30	54
493.4	32:1	31	34
495.4	32:0	76	100
519.5	34:2	34	23
521.4	34:1	84	85
545.5	36:3	26	11
547.4	36:2	100	69

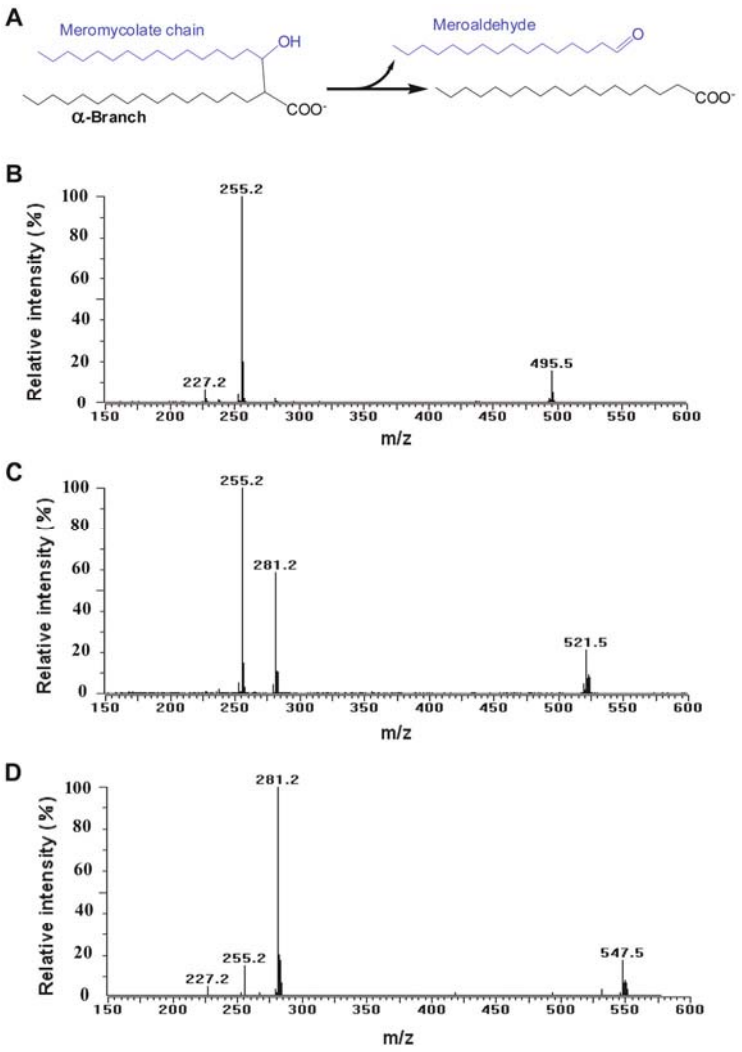

Fig. 3. Fragmentation analysis of the major [M-H]⁻ ions of mycolic acid in *C. glutamicum*. (A) Fragmentation processes proposed for the [M-H]⁻ ions of mycolic acid in *C. glutamicum*; (B) MS/MS spectrum of the [M-H]⁻ ion at *m/z* 495.5; (C) MS/MS spectrum of the [M-H]⁻ ion at *m/z* 521.5; (D) MS/MS spectrum of the [M-H]⁻ ion at *m/z* 547.5.

and the double bonds usually locate on the meromycolate chain.

Discussion

In this study, the structural diversity of mycolic acids in 5 different strains of *C. glutamicum* was characterized. The complexity of mycolic acids in *C. glutamicum* was observed not only by a wide range of masses but also by various structures found in each of the masses, that represent numerous isomers differing in the lengths of the meromycolate chain and α-alkyl group, as well as the unsaturated bonds. Many species of mycolic acid containing 20–40 carbons and 0–3 double bonds were observed in *C. glutamicum*, but the two hydrocarbon chains are similar in length. Mycolic acids in other *C. glutamicum* strains, such as *C. glutamicum* NCIB 10025, *B. lactofermentum* NCIB 9567, and *B. flavum* NCIB 9565 had been studied by Collins *et al.* (1982), but the detailed structural information on the mycolic acids was not determined due to the limited technology at the time. However, Collins *et al.* (1982) did point out that the predominant molecular species of mycolic acid in those *C. glutamicum* strains contained 30 or so carbons, consistent with our findings in 5 different *C. glutamicum* strains. In our study, more than twenty molecular species of mycolic acid were observed in all 5 strains of *C. glutamicum*, and their structures differ in the length and saturation of their fatty acid chains. The total number of carbons in mycolic acids isolated from each strain is in the range of 20–40 with 0–3 double bonds.

Mycolic acid in *C. glutamicum* observed here is quite different from the mycolic acids in *Mycobacterium tuberculosis*, which contain 76–87 carbons and unsymmetrical hydrocarbons. In *M. tuberculosis,* the α-alkyl group usually contains 22–24 carbons, while the meromycolate chain contains 54–63 carbons. The structural differences between mycolic acids in *C. glutamicum* and *M. tuberculosis* reflect the differences of the biosynthetic pathway in these bacteria. *Mycobacteria* have both FAS-I and FAS-II systems, which reflects their extremely complex lipid structure, while *C. glutamicum* has two FAS-I systems encoded by two large FAS-I genes (Puech *et al.*, 2000; Gande *et al.*, 2004; Radmacher *et al.*, 2005). There are different characteristics among the 5

strains of *C. glutamicum*, including production of different amino acids, but the composition and structure of their mycolic acids are similar. This suggests that the structure and biosynthesis pathway of mycolic acids is well conserved, and that the same approach could be used to modify the layer of mycolic acids in different strains of *C. glutamicum*. Changing the structure or composition of mycolic acids in *C. glutamicum* might improve the permeability of the cell wall, and thereby increase the production of amino acids.

Acknowledgements

Funding was provided by grants from National key Basic Research Program of China (2012CB725202), and the Basic Research Programs of Jiangsu Province (BK2009003).

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