Mutations in the PPE Genes that Confer Resistance to a Nitroimidazopyran Drug on *Mycobacterium bovis* Strains

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We used the IS1096 transposon to construct *Mycobacterium bovis* BCG mutants resistant to an antituberculosis drug PA-824 and isolated several different mutants. We identified the locations of the insertions and found that the insertions were at various sites including the genes for the PPE proteins. HPLC analyses of the extracts of these five PPE mutant cells showed that three mutants produced only F0, an intermediate for the synthetic pathway of coenzyme F$_{420}$ and the remaining two neither F0 nor F$_{420}$. These data suggest that the products of these PPE genes are somehow involved in the biosynthesis of the coenzyme F$_{420}$.

**Key words** — *Mycobacterium bovis*, transposon mutagenesis, PPE mutants, coenzyme F$_{420}$

The genus *Mycobacterium* includes many species pathogenic to humans and animals. *Mycobacterium bovis* is a member of the *M. tuberculosis* complex and remarkably similar to *M. tuberculosis*, the important agent of human tuberculosis, with regard to nucleotide sequence of the chromosomal DNA, cell physiology and pathogenicity[9]. *M. bovis* also causes tuberculosis in humans and animals.

Coenzyme F$_{420}$ is produced by both *M. tuberculosis* and *M. bovis*, and is required for activation of the experimental antituberculosis drug PA-824 by these bacteria[18]. F$_{420}$ functions as an electron carrier (Fig. 1) and is used by F$_{420}$-dependent glucose-6-phosphate dehydrogenase in both strains. Therefore, both strains incapable of producing F$_{420}$ are resistant to this drug at the concentration of 5 mg/ml. *M. bovis* and *M. tuberculosis* produce coenzyme F$_{420}$ and an intermediate, F0. It is thought that F$_{420}$-dependent reactions might be important for these pathogens. If it is true, specific enzymatic steps for the biosynthesis of F$_{420}$ could be targets for chemotherapy.

We constructed a mutant library of *M. bovis* BCG by using a mycobacterial transposon and isolated several different mutants resistant to PA-824. The insertion sites were identified and their DNA sequence was analyzed. We found that five of those mutants had insertions in the PPE genes. Three of the mutants produced only F0. Neither F0 nor F$_{420}$ was detected in the remaining two. The PPE gene family, together with the PE family, comprise approximately 10% of the coding capacity of the *M. tuberculosis* genome[7]. Although the role of the majority of PE and PPE proteins are unknown, there is some evidence that these proteins might be exposed to the cell surface and involved in antigenic variation[17]. We, in this article, report that five of the PPE proteins are involved in the synthesis of the coenzyme F$_{420}$, indicating that PPE proteins have enzymatic functions.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and media.**

*M. bovis* BCG Montreal strain and the plasmid pPR29 were described elsewhere[4,15]. *M. bovis* BCG cells were routinely grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) supplemented with 0.2% glycerol, 0.005% Tween-80 and 10% ADC enrichment.

**Electrotransformation of *M. bovis* BCG cells.**

*M. bovis* BCG cells were electrotransformed with pPR29 as described[15]. The transformed cells were plated on the Middlebrook 7H9 agar containing kanamycin (30 mg/ml) and gentamycin (5 mg/ml), and incubated at 32°C for 3 weeks. A colony was picked from the transformed cells, streaked on the same selection agar, and incubated for another 3 weeks. *M. bovis* BCG cells not transformed with pPR29 were always streaked parallel to the transformants as a negative control.

**Selection of the transposon-induced mutants resistant to PA-824.**

*M. bovis* BCG cells transformed by pPR29 were grown in...
Fig. 1. The structure of the coenzyme F_{420}. The oxidized form is shown on the left and the reduced form is shown on the right.

7H9 liquid medium and plated on the 7H9 agar plates with PA-824 (5 mg/ml), kanamycin (20 mg/ml), sucrose (2% w/v), riboflavin (0.05 mg/ml) and cyanocobalamin (0.2 mg/ml) at the cell density of 2.4×10⁵ CFU/plate. After incubation at 39°C for 3 weeks, we picked several colonies and streaked on the same selection agar, and incubated for another 3 weeks.

Inverse PCR and DNA sequence determination. 
The chromosomal DNA of the mutants was prepared[11], cleaved with EagI, self-ligated and used for inverse PCR (Choi et al. 2001). The PCR primers used were 5'-GTACGTCACCCT-GATGCTACGCT-3' and 5'-GAGTCAGGCTGATGCA-3', and both are complementary to the IS1096 DNA. The PCR products were purified by the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA) and the DNA sequence was determined at the University of Iowa DNA facility. The DNA sequence was analyzed by the BLAST program.

HPLC analysis.
Thirty milliliters of culture of each mutant was centrifuged and resuspended in 1 ml of distilled water. The cells were then incubated at 100°C for 15 minutes, centrifuged, and the supernatant was separated with Beckman System Gold 126 Solvent Module HPLC in the 300 mm×3.9 mm C18 column from Spelco Inc. at the elution rate of 1 ml/min. Buffer A was 275 mM sodium acetate (pH 4.7) containing 2% acetonitrile; buffer B was 100% acetonitrile. The portion of buffer B in the elution buffer was varied as follows: 0 to 2 min, 0%; 2 to 6 min, 0 to 2% (linear gradient); 6 to 15 min, 2 to 10%; 15 to 22 min, 28%; 22 to 27 min, 28% to 0%. The separated fraction was analyzed with System Gold 168 detector (Beckman Co.) and RF-10A XL fluorescence detector (Shimadzu Co.).

RESULTS

Construction of the *M. bovis* BCG insertional mutant library.
A mycobacterial transposon, IS1096, transposes at a frequency of 7.2×10⁵ per cell in a random fashion[6]. The plasmid, pPR29, carrying IS1096 was used to introduce insertional mutations to the *M. bovis* BCG strain. This plasmid carries a temperature-sensitive mycobacterial replication origin, a copy of the *Bacillus subtilis* sacB gene, and the resistance marker for gentamycin[14,15]. The transposon on the pPR29 carries a kanamycin marker on itself. The temperature-sensitive replication origin functions at 32°C but not at 39°C. The cells carrying the sacB gene are sensitive to 1% sucrose. BCG cells transformed with these plasmids were selected for resistance to kanamycin and sucrose to select for the cells carrying the transposon inserted in the chromosome but have lost the plasmid. Tens of colonies appeared under these selective conditions and several of them were analyzed further.

Identification of the transposon-inserted locations.
The cells of the insertional mutant library were subject to selection for resistance to PA-824. The chromosomal DNA was prepared from these cells and inverse PCR was performed to identify the insertion site of the IS1096 for each mutant. We obtained five different insertional PPE mutants and these were designated YMBCG34, 36, 45, 47 and 51, respectively.

Analysis of the mutants for the production of F_{420} and F₀.
The mutant cells were grown in liquid medium and their extract was analyzed by an HPLC. The *M. bovis* BCG wild
type extract showed distinct peaks for the F_{20} and F0, but the extract of the mutant cells showed various results (Table 1). Three mutants produced only F0 and showed higher peak for F0 than the wild type extract indicating the accumulation of F0. The remaining two mutants produced neither F_{20} nor F0.

**DISCUSSION**

Coenzyme F_{20} is a 7,8-dimethyl-8-hydroxy-5-deazaflavin and transfers electrons. It has been reported to be present in methanogens, mycobacteria, *Nocardia*, *Streptomyces*, a green alga *Scenedesmus*, a cyanobacteria *Synechocystis* and an halophilic archean *Halobacterium*.[4]. Although F_{20} is present among several organisms, there are minor differences in its structure and roles.

The biosynthetic pathway of the F_{20} is not understood clearly, but a hypothetical pathway had been proposed.[8,12,16] F_{20} is synthesized from F0 in accordance with their model. In this study, we isolated mutants producing only F0, but mutants producing only F_{20} were not isolated. This result coincides with their model.

Interestingly, mutants YMBGC34, 36, 45, 47 and 51 have the insertion in the PPE genes. There are 99 PE genes and 68 PPE genes in the *M. tuberculosis* genome.[2,7,19]. The product of these genes are peculiar proteins having conserved N-terminal sequence and variable C-terminus which is very rich for specific amino acids. The names PE and PPE are derived from the motifs Pro-Glu (PE) and Pro-Pro-Glu (PPE) found near the N-terminus of these proteins. Because of unknown function, it is now suggested that at least some of the PE and PPE proteins are surface proteins and play a role in adhesion and immune modulation.[2,3,5,13,17]. Vokuhl et al. reported that the synthesis of many PE and PPE proteins are regulated differentially.[20]. There has been no evidence reported for the enzymatic activities of the PE or PPE proteins yet. We, in this article, report for the first time that the insertional mutants in the PPE genes have lost the ability to produce the coenzyme F_{20}. Our data clearly suggest that at least some PPE genes encode products carrying enzymatic activities.

There is an interesting feature observed in the Table 2. The two smaller proteins, Rv2608 and Rv0096 have alanine as the most abundant amino acid and leucine as the second abundant. But, the other three larger proteins have glycine as the most abundant and asparagine as the second most abundant. Tekaia et al. reported the most abundant amino acid from the whole genome of the *M. tuberculosis* is alanine and the second is glycine.[19]. Alanine and glycine comprises 13.2% and 10%, respectively. PPE proteins in the Table 2 have alanine or glycine as the most abundant amino acids although their percentage is higher than the whole cellular percentage. Asparagine comprises only 2.5% for the whole *M. tuberculosis* cell, but the three large proteins in the Table 2 have much higher percentage of asparagines. Tekaia et al. suggested that asparagine storage could be a possible function for the PPE proteins.[19]. If that is true, Rv1917c, Rv0355c and Rv0305c appear to be dual function proteins: the first enzymes involved in biosynthesis of F_{20} and the second storage sites for asparagine. Sampson et al. expressed the Rv1917c protein in *M. smegmatis* and *M. bovis* BCG and found that this protein is surface exposed.[17].

Another intriguing feature is the unusually large size of the Rv0355c. This protein consists of 3,300 amino acids and the calculated molecular weight is 327 kD. We think this protein might be spliced after translation or its mRNA spliced before translation. Evidence is now accumulating for the protein splicing in prokaryotes.[1].

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**REFERENCES**

초록: *Mycobacterium bovis* 균주들이 nitroimidazopyran 항생제에 내성을 갖게 하주는 PPE 유전자들의 돌연변이들

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IS1096 transposon을 사용하여 결핵균군에 작용하는 항생제인 PA-824에 내성을 나타내는 *Mycobacterium bovis* BCG의 돌연변이를 연구로 하였고, 그 결과 24 종류의 서로 다른 돌연변이 균주들을 얻을 수 있었다. Transposon이 insertion된 부위를 알아내기 위해서 각각의 돌연변이 균주로 inverse PCR을 수행하였고, PPE 유전자를 포함한 여러 가지 부위에 insertion된 transposon의 위치를 확인할 수 있었다. PPE 유전자에 insertion 돌연변이가 발생한 5개 균주의 세포 추출물을 HPLC로 분석한 결과, 3개에서는 아생균주에서 관찰되는 coenzyme F_{420}가 존재하지 않았고, 그 생합성 경로의 중간산물인 F_{430}만 존재하였다. 또한 나머지 2개에서는 F_{420} 또는 F_{430} 이어도 존재하지 않았다. 이 결과는 PPE 유전자들의 산물들이 coenzyme F_{430}에 어떤 식으로든 관여하고 있음을 나타낸다고 할 수 있다.