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*fec*B, a gene potentially involved in iron transport in *Mycobacterium avium*, is not induced within macrophages

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Abstract

FecB is a protein involved in the transport of iron from ferric citrate in *Escherichia coli* and is present in the *Mycobacterium tuberculosis* genome sequence. Since the ability to retrieve iron from the host is crucial and may be related to virulence, we characterized the gene *fec*B from *Mycobacterium avium*, strain 101. An *E. coli*–mycobacterial shuttle plasmid with a *fecB*-promoter green fluorescence protein (*gfp*)-fusion was transformed into *M. avium* strain 104 to study the *fec*B-regulation. In vitro, the *fec*B expression in *M. avium* weakly correlated with the amount of iron present in the medium but the expression was maximal when there was no iron in the culture medium. In macrophages, *M. avium fec* B was not induced during the early phase of infection, suggesting that the iron concentration in the mycobacterial phagosome is not sufficiently low to stimulate the expression of *fec*B in *M. avium*.

Keywords: *Mycobacterium avium*; Ion transport; Phagosomes; FecB

1. Introduction

Mycobacterium avium is a slow growing, acid fast bacillus, commonly encountered in the environment, where it can be cultured from water and soil [1]. Mycobacterial diseases, e.g., tuberculosis and disseminated *M. avium* infections, are prevalent among AIDS patients [2,3]. Although the highly active antiretroviral therapy (HAART) has led to a decreasing incidence of almost all opportunistic infections in countries where this therapy is available [4], worldwide, the number of HIV-infected patients and of the resulting opportunistic infections, including tuberculosis and *M. avium* infections, are still rising [5]. In spite of recent advances in the field of mycobacteriology, the pathogenesis of these infections is poorly understood.

Once ingested by macrophages, *Mycobacterium tuberculosis*, as well as *M. avium*, is present within membrane-bound vacuoles that do not acidify or fuse with lysosomes [6–8]. Mycobacteria replicate inside these vacuoles, suggesting that they can obtain sufficient nutrients required for intracellular growth. The environment within phagosomes is likely to regulate gene expression in the bacterium, and therefore, it may influence bacterial growth and survival. Regulatory factors may be,

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for example, the presence, absence, or actual concentration of diverse particular elements. It has been shown that within the *Salmonella* vacuole in macrophages, the expression of *pho p*, a two-component regulator associated with virulence, is triggered by the low magnesium content [9,10].

Iron is an essential nutrient for all living cells and is a known regulator of virulence genes in bacteria [11]. It has been appreciated for a long time that iron is a risk factor for the development of tuberculosis [12] and necessary for the replication of mycobacteria [13]. The main iron acquiring molecules in mycobacteria are siderophores that either are excreted (exochelins and carboxymycobactins), or confined to the cell wall (mycobactins) where they may serve as an intermediate intraenvelope iron store [14]. Recently, we have shown that salicylate-derived siderophore-production is necessary for the retention of iron in the mycobacterial phagosome [15]. Several different siderophores have been described for other bacteria [16,17], but for mycobacteria it is unclear whether they produce or use different siderophores under iron limiting conditions. In the *M. tuberculosis* genome sequence, that has been deciphered by Cole et al. [18] the gene *fecB* (Rv 3044) had its possible function annotated by sequence homology to the *fecB* of *Escherichia coli*. FecB in *E. coli* belongs to the cluster #8 periplasmic solute-binding proteins [19] and is involved in the transport of iron from ferric citrate [20]. Its function in mycobacteria is so far unknown, but it is plausible to hypothesize that FecB may be involved in the iron-transport across the membrane during the process of infection and replication inside the phagosome. We therefore cloned the corresponding gene *fecB* from *M. avium* strain 101 (MAC101) and investigated its regulation by iron and during intracellular replication in macrophages.

2. Materials and methods

2.1. Bacterial strains and plasmids

M. avium strain 101 and strain 104 are clinical blood isolates from AIDS patients [21], *Mycobacterium smegmatis* mc^2 155 was kindly provided by William Jacobs, Jr. The *E. coli* strain XL1-Blue MRF' (Stratagene, La Jolla, CA) was used as the host for plasmid construction. Plasmids pK18 [22], pBluescript SK II+ (Stratagene) and pT7Blue-3 (Novagen, Madison, WI) were used for cloning and sequencing. Plasmid pFJS17, a derivative of pMV261 [23], is an *E. coli*–mycobacterial shuttle vector containing origins of replication for both organisms, a multi-cloning site upstream of the promoterless *gfpmutant* 2-gene [24] and a kanamycin resistance gene for screening transformants. pFJS17 was used to clone the *fecB*-promoter upstream of the gfp-gene (pDW6). A similar construct, pFJS8*gfpmut2*, which has the L5-promoter in front of the *gfpmutant* 2-gene, was used as a positive control in the studies of intracellular expression.

2.2. Molecular biological methods

A detailed description of DNA-preparation, polymerase chain reactions (PCR), generation of the *fecB*-amplicon probe, southern blot of genomic DNA, construction and screening of a small fragment library, cloning and sequencing procedures and bioinformatic informations on *fec*B are given in the online available supplemented material at the publishers webpage.

2.3. Transformation of MAC104 and analysis of transformed clones

MAC104 was transformed as described previously [25,26]. In order to reanalyze plasmids of transformed colonies, plasmid DNA was extracted together with genomic DNA and retransformed into *E. coli*. The presence of pDW6-DNA was confirmed by comparison of a digestion-map with different restriction enzymes.

2.4. Preparation of iron-containing media

The glass and plastic ware that was used was washed with metal-free detergent (Acationox®, Baxter) and extensively rinsed with distilled and deionized water thereafter. Distilled and deionized water that was treated with Chelex100 resin (BioRad) and filtered thereafter was used as a base for all media. All media were stored in plastic ware in order to avoid leaching of iron from glass surfaces. The media were prepared according to the recipe of 7H9 (Difco Laboratories, Detroit, MI) with addition of 0.1% glycerol omitting the ferric ammonium citrate, which was added thereafter. After sterilization and addition of OADC (10 ml/100 ml of medium) and kanamycin (400 μg/ml), the iron concentration was confirmed by the ferozine assay (Sigma, St. Louis, MO), with a detection limit of 1 μM and above in our hands. OADC has an iron concentration of ~3 μM, so the minimal concentration of iron in a respective medium was ~0.3 μM.

2.5. Expression of fecB at different iron concentrations

MAC104::pDW6 were grown on regular 7H11 plates (hereafter referred to as "iron supplemented") and then exposed to media containing the different iron concentrations for up to 14 days at 37 °C in the shaking water bath. Dilutions were plated on 7H11 containing 400 μg/ml kanamycin and the colonies counted after 10 days. *M. smegmatis*::pDW6 was grown in media containing kanamycin 50 μg/ml and Tween 80 (0.1%) for up to 10 days at 37 °C in the shaking water bath, dilutions plated