INFECTION AND IMMUNITY, Jan. 2007, p. 504–507 Vol. 75, No. 1
0019-9567/07/$08.00+0 doi:10.1128/IAI.00058-06

Signature-Tagged Transposon Mutagenesis Identifies Novel *Mycobacterium tuberculosis* Genes Involved in the Parasitism of Human Macrophages▽

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Received 11 January 2006/Returned for modification 28 February 2006/Accepted 26 September 2006

Using signature-tagged transposon mutagenesis, we isolated 23 *Mycobacterium tuberculosis* mutants, corresponding to 21 genes or genetic regions, attenuated in their ability to parasitize human macrophages. Mutants disrupted in the ABC transporter-encoding genes Rv0986 and Rv0987 were further characterized as being impaired in their ability to bind to host cells.

Parasitism of host macrophages (Mϕs) by pathogenic mycobacteria, including *Mycobacterium tuberculosis*, the agent of tuberculosis in humans, is a key feature of mycobacterial virulence that needs to be further understood (7, 11, 25). Mϕ infection by the bacillus involves adhesion to and phagocytosis-mediated entry inside the host cell, as well as resistance to phagosome-lysosome fusion and to free radicals (25). A better comprehension of the microbial molecular determinants involved in these processes might help not only to better understand the cell biology of mycobacterial infections but also to design novel targets for new antimycobacterials and possibly better vaccine candidates. Various approaches have been used in the past to identify mycobacterial genes involved in macrophage infection. These approaches include promoter fusion to reporter genes in order to identify genes induced intracellularly (6, 31), substractive hybridization techniques (8, 21), and transcriptome (27) and proteome (12, 16) analyses, as well as screening of mutant libraries (4, 18, 20, 28). Here we took advantage of the negative selection technique signature-tagged transposon mutagenesis (STM) (9) to identify virulence genes required for *M. tuberculosis* parasitism of human Mϕs.

Screening of an *M. tuberculosis* STM library in human Mϕs. Mycobacteria were grown at 37°C in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Difco Laboratories) and 0.05% Tween 80 (Sigma-Aldrich, Corp., St. Louis, MO). THP-1 (ATCC TIB-202) cells were grown in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Dutscher, Brumath, France). THP-1 monocytes were differentiated into Mϕs by incubation with 10 ng of phorbol 12-myristate 13-acetate (Sigma)/ml for 48 h. An STM library of 1,410 members was constructed with *M. tuberculosis* MT103 as a parental strain (4) and was used to infect THP-1 Mϕs for 7 days (input pools). Bacteria were extracted from infected cells and used in a new 7-day infection round in order to enrich in attenuated mutants. Transposon-mediated *M. tuberculosis* mutants were cultured in the presence of kanamycin (20 μg/ml; Sigma). To dislocate clumps, bacteria were passaged through a needle and mildly centrifuged before infection, as described previously (24, 29). Infected cells were lysed after each infection round, and bacteria were recovered by plating the material onto selective agar medium. Mutants were recovered 3 weeks after culture (output pools), and the chromosomal DNA was extracted as described previously (17). Tags from input and output pools were amplified and hybridized as described previously (4). From the initial 1,410 mutants, 32 were selected as candidate attenuated clones after two 7-day infection rounds. Of the 32 candidates, 23 were firmly confirmed as attenuated after individual infection of THP-1 Mϕs and comparison of doubling times with the wild-type strain over a 7-day period (Table 1). Transposon insertion sites were identified by ligation-mediated PCR and sequencing as described previously (19). The identified virulence genes (Table 1) included genes potentially involved in lipid metabolism and cell wall biosynthesis (*papA1*, *mmpL2*, *pks6*, Rv2958c, *alkB*, and *drrB*), as well as genes involved in intermediary metabolism and metal utilization (*bfrB*, *moaC1*, *moaX*, Rv1817, and Rv0097), genes of the PE/PPE family (*ppe5* and *ppe8*), and genes of yet-unknown function (Rv2227, Rv2336, Rv1502 and Rv1503c, Rv2104c, and Rv2954c and Rv2955c). Interestingly, some of these genes (*mmpL2*, *drrB*, Rv0986, and Rv2336) have been suggested to play a part in mycobacterial virulence in previous studies based on various in vivo and in vitro screening approaches (4, 18, 26). This reinforces the relevance of the STM technology to search for virulence genes in *M. tuberculosis*.

Rv0986 and Rv0987 disruption reduces the ability of *M. tuberculosis* to bind to host cells. One additional attenuated mutant carried a transposon insertion immediately after nu-

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▽ Published ahead of print on 9 October 2006.

TABLE 1. Attenuated *M. tuberculosis* mutants identified by STM

Mutant	Disrupted ORF[a]	Putative function	Growth index[b]	Other studies[c]
28AD1	*mmpL2*	Fatty acid transport	0.7	4
38AA2	Rv0097	Possible oxido-reductase	0.7	
28AA4	Rv0986	ABC transporter	0.7	18
31AC3	Rv2227	Unknown	0.6	
36AB4	Rv1817	Flavoprotein	0.6	
36BF6	*drrB*	Phthiocerol dimycocerosate transport	0.6	26
38AC3	Rv2954c-Rv2955c*	Unknown-unknown	0.6	
28AA2	*yrbE4A*	Part of *mce4* operon	0.5	26
27BB3	*bfrB**	Iron metabolism (bacterioferritin)	0.4	
31AA2	*moaC1*	Molybdopterin synthesis	0.4	
36AF2	Rv1502	Unknown	0.4	
27AC6	*alkB*	Fatty acid metabolism	0.3	
28AB3	*papA1*	Sulfolipid synthesis	0.3	
35AA4	*ppe5*	PE/PPE family	0.3	
36AC2	*pks6*	Polyketide synthase	0.3	
36AC3	*ppe8*	PE/PPE family	0.3	
28BG4	Rv2336	Unknown	0.2	18
29BG6	Rv2104c-Rv2105*	Unknown-IS*6110* transposase	0.2	
31AG4	Rv2336	Unknown	0.2	
34AG4	Rv2958c*	Glycosyltransferase	0.2	
36AF4	*moaX*	Molybdopterin synthesis	0.2	
38AB3	Rv1502-Rv1503c*	Unknown-unknown	0.2	
27AA2	*ppe5*	PE/PPE family	0.2	

[a] ORF, open reading frame. *, A Tn insertion occurred upstream of the starting site or in between the two genes.
[b] The growth index was calculated as the doubling time of wild-type *M. tuberculosis* divided by that of the mutant in THP-1 cells over a 7-day period.
[c] The genes have been previously identified in other genetic screenings in vivo or in vitro.

cleotide T_{554} in Rv0986, with the transposon (Tn) kanamycin resistance gene oriented in the same direction as the Rv0986 gene. Rv0986 has been recently identified as playing a part in the inhibition of phagosome-lysosome fusion through a screening approach at the subcellular level (18). It encodes a putative ATP-binding protein sharing high similarity with the *Agrobacterium tumefaciens* AttE polypeptide and predicted to form an ABC transporter together with Rv0987 (3). In *A. tumefaciens*, an α-proteobacterium responsible for crown gall in plants, the plasmid-borne *attE-H* genes are thought to encode an ABC transporter involved in the secretion of a host cell adherence factor (13, 14). We have recently shown that the Rv0986-8 gene cluster arose through horizontal gene transfer in the ancestor of *M. tuberculosis* (22). The individual infection profile of the Rv0986::Tn mutant in THP-1 Mϕs (Fig. 1A) suggested that this gene might be involved in mycobacterial adherence to host cells. In order to validate this phenotype, cold binding assays (30) were realized using the Rv0986::Tn mutant. In addition, a Rv0987::Tn mutant generated independently by transposon mutagenesis for the purpose of another study (10) and carrying a Tn insertion immediately after nucleotide C_{1592} was included in the experiments. Cells were infected in triplicates for 4 h at 4°C in RPMI 1640 at a multiplicity of infection of one bacterium per cell, extensively washed in phosphate-buffered saline, lysed, and plated onto agar medium for CFU scoring. The Rv0986::Tn and Rv0987::Tn mutants were found to be affected in their ability to bind to THP-1 Mϕs (Fig. 1B). Binding of the Rv0986::Tn mutant was reduced by 51.5% ± 11.8% ($n = 4$) compared to that of the wild-type strain. This phenotype could be restored after complementation using pYUB412-derived cosmids (1) (Fig. 1B). The MTCI229 and MTCI310 cosmids used in the study carry 38.7- and 20.8-kb DNA fragments covering the 1077.0- to 1115.7-kb and 1086.5- to 1107.3-kb regions of the *M. tuberculosis* chromosome. These cosmids encompass the Rv0986-8 operon (1101.8 to 1106.3 kb, genomic coordinates). The binding default of the two mutant strains was confirmed at 37°C using 2 μg of cytochalasin D

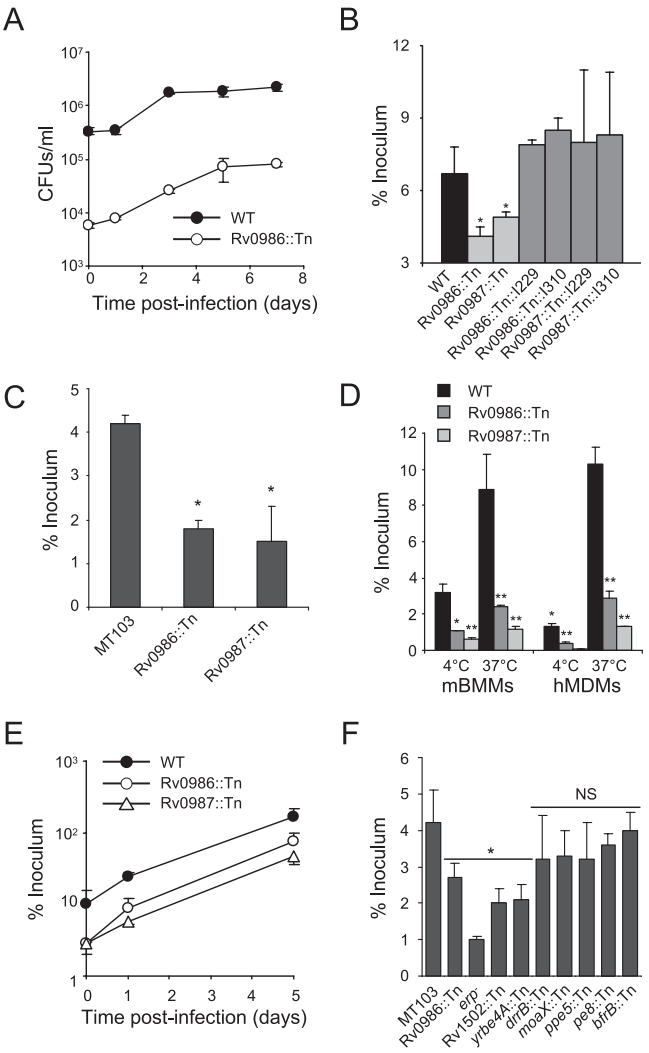

FIG. 1. Rv0986-8 plays a key role in *M. tuberculosis* binding to host cells. (A) Infection profile of *M. tuberculosis* wild-type and Rv0986::Tn strains in THP-1 Mϕs. (B) Binding capacity of *M. tuberculosis* wild-type (WT), Rv0986::Tn, Rv0987::Tn, and MTCI229- and MTCI310-complemented mutant strains to THP-1 Mϕs. Cells were infected for 4 h at 4°C. (C) Binding capacity of *M. tuberculosis* wild-type (WT), Rv0986::Tn, and Rv0987::Tn strains to THP-1 Mϕs. Cells were infected for 4 h at 37°C in the presence of cytochalasin D. (D) Binding capacity of *M. tuberculosis* wild-type (WT), Rv0986::Tn, and Rv0987::Tn strains to mouse bone marrow-derived Mϕs (mBMMs) and human monocyte-derived Mϕs (hMDMs). Cells were infected for 4 h at 4°C or at 37°C. (E) Intracellular survival of *M. tuberculosis* MT103 wild-type (WT), Rv0986::Tn, and Rv0987::Tn strains in human MDMs. (F) Binding capacity of *M. tuberculosis* wild-type (WT), Rv0986::Tn, *erp*-deficient, Rv1502::Tn, *yrbE4A*::Tn, *drrB*::Tn, *moaX*::Tn, *ppe5*::Tn, *ppe8*::Tn and *bfrB*::Tn strains to THP-1 Mϕs. Cells were infected for 4 h at 4°C. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test of median comparison).

(Sigma)/ml in order to block phagocytosis (Fig. 1C) and was also observed in human blood monocyte- and murine bone marrow-derived Mϕs prepared as described previously (24, 29) (Fig. 1D), as well as in nonphagocytic cells such as epithelial cells and fibroblasts (data not shown). Once inside phagocytes, when grown at 37°C, the mutants were not significantly impaired in their ability to replicate compared to the wild-type strain (Fig. 1E). This result seems at odds with that from another study (18), in which a Rv0986-deficient mutant was found to be impaired in its ability to block phagosome-lysosome fusion and to replicate inside Mϕs. This difference between the two studies might be due to the different contexts in which the mutants were generated. We used *M. tuberculosis* MT103 as the parental strain, whereas Pethe et al. (18) used CDC1551 to construct their mutant.

Analysis of the secreted products in *M. tuberculosis* wild-type and Rv0987::Tn strains. The results above raised the possibility that the Rv0986-7 transporter might secrete an adherence factor that allows bacteria binding to macrophages and other cell types. Analysis of the lipid and sugar contents in the cell envelope, as well as in the secreted products, revealed no major difference in the amount and structure of methyl-branched fatty acid-containing acyltrehaloses, cord factor, phospholipids, phosphatidylinositolmannosides, phthiocerol dimycocerosates, mycolic acids, glucan, arabinomannan, mannan, and lipoarabinomannan between the Rv0987::Tn mutant and the wild-type strains (data not shown). This suggests that the Rv0986-7 transporter may carry an as-yet-unidentified protein, polysaccharide or (glyco)lipid, which will require further examination.

Another possible explanation is that Rv0986 and Rv0987 disruption might alter the architecture and integrity of the mycobacterial cell wall, which is likely to affect the adherence properties of the bacillus. In order to assess how common was the adhesion defect among the mutants we have isolated, we have repeated the binding assay using a cohort of these mutants, together with another attenuated mutant inactivated in the *erp* gene encoding a cell surface virulence factor (2). As shown in Fig. 1F, binding of the Rv0986-, *erp*-, *yrbE4A*-, and Rv1502-deficient mutants was found to be significantly altered compared to that of the wild-type strain, whereas binding of the other strains tested (deficient in *drrB*, *moaX*, *ppe5*, *pe8*, and *bfrB*) was not. Diminished binding of the *erp*-deficient strain to host cells has been observed previously (J.-M. Reyrat, unpublished data). The *yrbE4A* gene is part of the *mce* operons that are involved in host cell entry. Mce1 has been shown to promote adhesion of latex beads to HeLa cells (5), and Mce4A (Yrbe4A) has been suggested to have a similar property (15). Both *erp* and *mce* genes encode products located in the cell envelope of the bacillus. Alteration of the cell envelope architecture is likely to affect the ability of the strain to bind to cells, which has also been observed in other mutants of the cell envelope (23). The reduced ability of the Rv0986- and Rv0987-deficient mutants to bind to host cells might thus be the consequence of any alteration of the cell envelope architecture in these strains rather than of the lack of an adherence product secreted by the operon.

Rv0986 and Rv0987 disruption does not reduce *M. tuberculosis* virulence in vivo. In order to evaluate the phenotype of the two mutants in vivo, we infected 6- to 8-week-ld female C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) intranasally with 1,000 CFU of the wild type or mutant strains, and we collected lungs and spleens 1, 21, and 42 days after inoculation for bacterial load measurement (Fig. 2). Although statistically significant, the reduction in CFU of the mutants observed in the lungs of the animals 21 days after infection probably did not result from a genuine in vivo attenuation but rather from lower infecting doses (see the bacterial loads at day 1). This difference was not observed in the spleen at either time point and was even reversed in the lungs at the later time point observed (day 42). Histological examination of the lungs did not reveal any marked differences in inflammation and granuloma formation between the wild-type and the mutant strains (data not shown). Since the Rv0986::Tn and Rv0987::Tn mutants show a cell-binding defect, this result raises the possibility that reinfection of host cells in vivo might not be required for sustaining the infection over time, which will require further investigation.

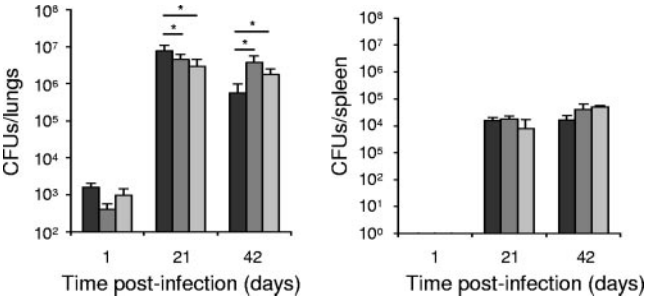

FIG. 2. Rv0986-8 is not involved in *M. tuberculosis* host tissue colonization in vivo. C57BL/6 mice were infected intranasally with 1,000 CFU. Lungs (left panel) and spleens (right panel) were collected at days 1, 21, and 42 postinfection, and bacterial loads were measured. *, $P < 0.05$ (Mann-Whitney test of median comparison).

In conclusion, the present study describes novel *M. tuberculosis* genes involved in Mϕ parasitism and demonstrates this virulence process to be a multifactorial phenomenon. In addition to its possible role in early phagosome remodeling (18), the Rv0986-7-encoded ABC transporter is likely to be involved in host cell binding either through secretion of an as-yet-unidentified adherence factor or through maintaining the architecture and integrity of the mycobacterial cell envelope. Future studies will seek to decipher the exact role of the various virulence factors, including Rv0986 and Rv0987, identified through this STM-based approach in *M. tuberculosis* pathogenesis.

This work was supported by Institut Pasteur and the European Community (contracts QLK2-CT-2001-02018 and QLK2-CT-2000-01761). V.R.M. is a fellow of Conacyt.

We thank D. G. Russell (Ithaca, NY), G. R. Stewart (London, United Kingdom), and R. Brosch (Paris, France) for helpful discussions and critical reading of the manuscript and P. Charles (Paris, France) for excellent technical assistance in histology. We thank Y. Bordat and N. Maeda (Paris, France) for excellent technical help with the STM.

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Editor: J. L. Flynn