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Macrophage-Induced Genes of *Salmonella Typhimurium*

Abstract

We developed an effective strategy for the systematic identification of macrophage-induced genes of *S. typhimurium*. MudJ transposon-insertion mutagenesis was used to create a library of random *lacZ* fusions in *S. typhimurium* strain ATCC 14028. Insertions in macrophage-induced genes were identified by comparing the ability of individual MudJ mutants to ferment X-gal in either the presence or the absence of the murine macrophage cell-line J774. Screening of 940 mutants resulted in the identification of ten MudJ mutants with insertions in macrophage-induced genes. When tested for virulence, 6 out of these 10 mutants were macrophage-sensitive and 3 were avirulent in mice. Southern blot- and sequence analysis of the cloned macrophage-induced genes indicated that all ten MudJ insertions were in different genes. Some genes were found to be specific for *Salmonella* while others are conserved in most pathogenic bacteria. One mutant had a MudJ insertion in the enterotoxin gene *stn*, a known *S. typhimurium* virulence factor, the others are in new virulence genes.

Introduction

When *Salmonella typhimurium* infects a host it is exposed to various environmental changes, such as changes in temperature, nutrient supply, osmolarity and pH (Groisman *et al.*, 1990; Finlay and Falkow, 1988; Fields *et al.*, 1986; Miller *et al.*, 1989; Mekalanos, 1992). In addition the pathogen is exposed to oxygen radicals and defensins produced by the hosts macrophages (Buchmeier and Heffron, 1990). To successfully adapt to this new hostile environment within the

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host, differential expression of bacterial genes must occur. We here describe the development of an effective strategy (Macrophage-Induced Promoter Assay; MIPA) for the systematic identification of *S. typhimurium* genes that are highly expressed when the bacteria are in contact with macrophages but not when they are absent. Use of this MIPA resulted in the identification of 10 macrophage-induced loci of *S. typhimurium.*

Screening for mutants with MudJ insertions in genes of which the expression is altered upon contact with macrophages

MudJ transposon-insertion mutagenesis was used to create a library of random *lacZ* fusions in *S. typhimurium* strain ATCC 14028. From this library we selected 940 individual mutants and screened them for their ability to express *β*-galactosidase in the presence and absence of J774 macrophages. Only MudJ transposon insertions in actively transcribed genes will result in the production of *β*-galactosidase. The production of this enzyme can easily be established by its potential to ferment the colourless sugar X-gal (5-bromo-4-chloro-3-indolyl-*β*-galactose) into an intense blue product. MudJ mutants in macrophage-induced genes can therefore be identified as those mutants that ferment X-gal (i.e. turn blue) in the presence of J774 cells but not in their absence.

Of the 940 individual MudJ mutants tested with the above method, ten showed significant *lacZ* production when in contact with J774 cells while no detectable *lacZ* production was observed when these mutants were grown in tissue culture medium alone. Of the remaining 930 mutants, 718 were white both in the absence and presence of J774 cells, 212 mutants were blue under both conditions. None of the MudJ mutants were blue in the absence of cells while white in their presence.

Analysis of the MudJ mutants that showed macrophage-induced β-galactosidase expression

Although screening of the MudJ libraries did not result in the identification of mutants with MudJ insertions in macrophage repressed genes, 10 MudJ mutants with insertions in genes that are specifically induced upon invasion of macrophages were found. To exclude the possibility that the macrophage-induced phenotype of these mutants was the result of a MudJ insertion in a gene required for the uptake or synthesis of a nutrient lacking inside macrophages all ten MudJ mutants were tested for growth on M9 minimal agar containing X-gal. All mutants grew and none turned blue on these plates. Niether did anaerobic growth, growth at 42°C, or low pH, result in the production of *β*-galactosidase.

Southernblot analysis was used to 1) exclude that the MudJ insertions were all in the exact same location of the genome, and to 2) confirm that these mutants contained only a single copy of the MudJ transposon. The DNA from

all 10 MudJ mutants was analysed with three different restriction endonucleases (*Alu* I, *Hae* III, and *Taq* I). Upon digestion the DNA was separated on a 1.5% agarose gel, blotted to Hybond-N and probed with the left-hand end of the MudJ transposon. All mutants contained only a single copy of the MudJ transposon and each mutant showed a unique hybridisation pattern (data not shown). This indicates that these 10 macrophage-induced mutants are in different genes and therefore probably define 10 different macrophage-induced loci on the salmonella genome. Data on the genomic map position of the MudJ insertions confirms this assumption.

To further characterize these mutants, inverse PCR was used to clone the genomic DNA flanking the MudJ transposon. Southern blot analysis was used to determine the species specificity of the cloned flanking DNA. The cloned DNA was labelled and tested against *Eco* RI digested DNA from *S. typhimurium* (5 independent isolates tested), *Salmonella enteritidis*, *Salmonella dublin*, *Salmonella typhi*, *Salmonella arizona*, *Salmonella heidelberg*, *Salmonella infantis*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella boydii*, *Shigella sonnei*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, enterohemolytic *Escherichia coli*, enteropathogenic *Escherichia coli*, enterotoxigenic *Escherichia coli*, enteroinvasive *Escherichia coli*, *Proteus mirabilis*, *Vibrio cholerae*, *Erwinia carotovora*, *Klebsiella pneumoniae*, and *Pasteurella haemolytica*. Three basic patterns were observed (Table 1): 1) the gene is exclusively present in *Salmonella typhimurium*, 2) a homologous gene was present in all Salmonella serotypes, but not in any of the other bacteria, and 3) homologue hybridisation signals were detected in (almost) all species.

The nucleotide sequence of the cloned DNA was determined and the obtained sequence was compared to the sequences in the databases using the BLAST programs as provided by NCBI (Altschul *et al.*, 1990). The BLAST-search data are summarized in Table 1.

Table 1. Sumary of data on macrophage-induced genes.

Mutant	Species specificity	Sequence homology	Virulent in
1	Non-specific	None	mice only
2	Salmonella specific	*E. coli rbsR**	mice only
3	Salmonella specific	None	mice only
4	Salmonella specific	*E. coli poxB*	mice only
5	Salmonella specific	*S. typhimurium stn*	mice only
6	*S. typhimurium* specific	None	mice only
7	Salmonella specific	None	macrophages & mice
8	Salmonella specific	*araD**	macrophages only
9	Non-specific	None	macrophages only
10	Salmonella specific	None	macrophages only

*See text for explanation.

To estabish if these macrophage-induced genes represent virulence factors, we tested the virulence of these mutants both *in vitro* (proteose-peptone elicited peritoneal macrophages) and *in vivo* (oral inoculation in mice). With the exeption of mutant #7 all mutants revealed significant reduction of virulence (Table 1).

Discussion

Several studies demonstrated that adherence to and invasion of host cells results in the *de novo* synthesis of *S. typhimurium* proteins (Finlay *et al.*, 1989; Buchmeier and Heffron, 1990; Aranda *et al.*, 1992; Fierer *et al.*, 1993). It was demonstrated that the contact between *Salmonella* and the host-cell results in the increased synthesis of at least 30 bacterial proteins (Abshire and Neidhardt, 1993; Buchmeier and Heffron, 1990). This *de novo* bacterial protein synthesis seems essential for successful invasion of host cells (Finlay *et al.*, 1989; MacBeth and Lee, 1993; Kusters *et al.*, 1993; Ernst *et al.*, 1990; Schiemann and Shope, 1991). Almost no data exist on the specific host-cell related triggers that cause the induction of *S. typhimurium* virulence-genes. The best studied stimulus known to induce the production of invasion related proteins in the absence of cells is growth under low-oxygen conditions but many other stimuli are probably involved as well (Lee and Falkow, 1990; Ernst *et al.*, 1990; Schieman and Shope, 1991, Jones and Falkow, 1994). Carbon starvation and growth state are some of the factors known to indirectly induce virulence genes by the action of the alternative sigma-factor *katF* (Coynault *et al.*, 1992; Kowarz *et al.*, 1994).

In spite of their importance, only a few of the *Salmonella typhimurium* proteins that are induced upon contact with eukaryotic cells have been identified. They include common stress response proteins like RecA, RecBC, GroEL and DnaK, as well as the virulence related proteins SpvR SpvABCD, PagC and InvE (Buchmeier and Heffron, 1990; Abshire and Neidhardt, 1993; Buchmeier *et al.*, 1993; Miller *et al.*, 1992). Identification and characterization of additional host-induced genes might provide clues on how this bacterium senses its host. Mahan *et al.* have designed an elegant method (IVET) for the identification of host-induced genes *in vivo* (Mahan *et al.*, 1993, 1994). Their method is based on the complementation of a *purA*$^-$ *Salmonella* with a promoterless *purA-lac* construct fused to chromosomal fragments of *Salmonella typhimurium.* However, this method will only identify genes that are induced during all stages of the infection. Repressed genes and genes that are induced only during a brief stage of the infection process can not be identified with this method.

In an attempt to identify the genes missed by the IVET we designed the MIPA (Macrophage-Induced Promoter Assay) as a simple *in vitro* assay for the detection of genes that are induced or repressed during (one particular stage of) the infection. In the MIPA individual mutants, with transposon generated transcriptional *lacZ* fusions, are used to infect macrophages. Following the infection, cells and bacteria are overlaid with X-gal containing agar and screened for blue

staining. Comparison of the blue staining of bacteria that are in contact with macrophages with the staining of mutants grown in the absence of cells will identify mutants in which *lacZ* is fused with host-cell regulated genes.

Macrophage induction was demonstrated with 10 mutants from a total of 940 randomly selected MudJ mutants. Since all mutants map at distinct locations in the genome, show distinct hybridization patterns with all three restriction enzymes used, and none of the sequences indicates that two or more insertions are in the same gene or operon, they must represent MudJ instertions in 10 different macrophage-induced loci. None of the MudJ insertions resulted in an auxotrophic phenotype, nor did anaerobic growth or growth at 42°C, low pH, or on minimal agar result in the production of β-galactosidase. This indicates that the observed induction is macrophage specific.

Testing of the MudJ mutants did not result in the identification of mutants that showed macrophage-induced repression of *lacZ*. Maybe such mutants were not present in our library, alternatively, the stability of the β-galactosidase may require infections at a lower MOI and more rigorous washes to remove background staining. Since the MIPA does not require expensive equipment, or the availability of an animal model-system, and since a wide variety of transposons exist for the creation of transcriptional-fusion mutants in pathogenic bacteria, the MIPA is a simple, but powerfull method for the identification of host-induced genes in various bacterial pathogens.

Interestingly all but one mutant show some sort of attenuation. This is indicative of host-induced genes being virulence factors. Sequence analysis of the macrophage-induced genes reveals that the majority of the MudJ insertions is in new virulence genes for which no known homologues exist in any of the databases. Only the the MudJ insertion of mutant #5 was found to be in a known virulence factor; the *S. typhimurium* enterotoxin *stn* (Chopra *et al.*, 1994). Chopra and coworkers were puzzeled by their observation that the *stn* gene is not expressed under normal laboratory conditions. Our finding that this gene is macrophage-induced accounts for their finding.

Although not a known virulence factor, a second gene that was unambiguously identified by its homology to sequences in the databases was the MudJ inactivated gene from mutant #4. The sequence derived from this gene was almost identical to the pyruvate oxidase gene (*poxB*) of *E. coli*. Identity was confirmed by the genomic map position of the MudJ insertion which corresponds with the published location of *poxB*. We can only guess why this gene is induced by macrophages, maybe it serves some function in the elimination of oxidative membrane damage caused by the macrophage.

The sequence of mutant #8 was found to be homologous to the *araD* gene. The macrophage-induced gene disrupted by the MudJ insertion is a homologue of the *araD* gene and not *araD* itself. The MudJ disrupted gene is located at 80′ on the genomic map, while the *araD* gene is located at 2′ on the *S. typhimurium* genome. A similar gene duplication of the *araD* gene is known to exist in the genome of *E. coli*. In both bacteria the function of the protein encoded by this *araD* homologue is unknown.

The sequence of mutant #2 shows strong homology to the repressor of the ribose operon (*rbsR*) of *E. coli.* In addition some weaker homologies to other repressor genes were found. As with mutant #5 this gene does probably not correspond to the *rbsR* gene but more likely it is a homologous *rbsR*-like gene.

We are currently characterizing the MIPA positive mutants in a quantitative assay to establish the expression levels at different time points upon infection of eukaryotic cells and mice. This will eventually provide insight into the host- related factors that cause the specific host related induction of bacterial virulence genes.

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