Vaccine 28 (2010) 940–949

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Salmonella enterica serovar Typhimurium mutants completely lacking the F_0F_1 ATPase are novel live attenuated vaccine strains

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ARTICLE INFO

Article history:
Received 22 July 2009
Received in revised form 27 October 2009
Accepted 29 October 2009
Available online 3 December 2009

Keywords:
Salmonella Typhimurium
F_0F_1 ATPase
Vaccination

ABSTRACT

The F_0F_1 ATPase plays a central role in both the generation of ATP and the utilisation of ATP for cellular processes such as rotation of bacterial flagella. We have deleted the entire operon encoding the F_0F_1 ATPase, as well as genes encoding individual F_0 or F_1 subunits, in *Salmonella enteric* serovar Typhimurium. These mutants were attenuated for virulence, as assessed by bacterial counts in the livers and spleens of intravenously infected mice. The attenuated *in vivo* growth of the entire *atp* operon mutant was complemented by the insertion of the *atp* operon into the *malXY* pseudogene region. Following clearance of the attenuated mutants from the organs, mice were protected against challenge with the virulent wild type parent strain. We have shown that the F_0F_1 ATPase is important for bacterial growth *in vivo* and that *atp* mutants are effective live attenuated vaccines against *Salmonella* infection.

1. Introduction

Salmonella enterica is a diverse pathogen classified into >2400 serovars and is the cause of important infections in both humans and livestock. *S. enterica* serovar Typhi (*S.* Typhi) is the causative agent of typhoid fever, a serious systemic disease in humans. It is estimated that there are 22 million cases of typhoid fever annually worldwide, resulting in 200,000 deaths [1,2]. Vaccination against *S.* Typhi is a potentially attractive method of disease control, but current vaccines have significant drawbacks and there is a need for improved versions [3,4]. *S. enterica* serovar Typhimurium (*S.* Typhimurium), a common cause of gastroenteritis (salmonellosis) in humans, has added significance because infection of mice with this serovar generates a systemic infection with important similarities to human typhoid fever. This mouse model has been used extensively to study typhoid-like infections [5,6].

The F_0F_1 ATPase is a complex of membrane proteins found in eukaryotes and prokaryotes that has been best studied in mitochondria [7,8], chloroplasts [9,10] and *Escherichia coli* [11–13]. It plays a central role in energy transduction, generating ATP from ADP and Pi substrates via oxidative phosphorylation. The synthesis of ATP is driven by the flow of protons into the cell, generating a proton motive force which energises processes such as motility and active transport [14–17]. In *E. coli*, the genes encoding the F_0F_1 ATPase are located in a single operon, *atpIBEFHAGDC*, transcribed from a promoter upstream of *atpI* [18–20]. The F_0 subunit of the ATPase is a hydrophobic membrane-embedded proton channel encoded by genes *atpBEF*. The F_1 subunit constitutes the catalytic ATPase, encoded by *atpHAGDC* [19,21]. The first gene in the operon, *atpI*, has no defined function and does not appear to form part of the F_0F_1 ATPase complex [22]. This genetic organisation is conserved between *E. coli* and *S.* Typhimurium.

A comprehensive identification of genes required for *S.* Typhimurium infection of mice by our laboratory identified mutation of *atpA* as an attenuating lesion [23]. A defined *atpA* deletion mutant was subsequently confirmed to be attenuated for growth *in vivo* and furthermore was found to offer significant protection against subsequent challenge [23]. Here we present a full analysis of the role of the F_0F_1 ATPase in *S.* Typhimurium infection and the potential use of mutants in the *atp* operon as live attenuated vaccines.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. Bacteria were grown at 37 °C in Luria–Bertani (LB) broth or on LB agar. Media were supplemented with antibiotics where stated, at the following concentrations, kanamycin 50 μg/ml, ampicillin 100 μg/ml and chloramphenicol 25 μg/ml. Minimal medium (used to determine carbon source utilisation) consisted of M9 salts

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doi:10.1016/j.vaccine.2009.10.146

Table 1
Salmonella strains and plasmids used in this study.

Bacterial strain or plasmid	Description	References
SL1344	Wild type parent for this work, mouse-virulent strain of *S.* Typhimurium	[43]
LB5010	*S.* Typhimurium. LT2 *galE* mutant, r^{-} m^{+}	[44]
SL3261	*aroA* mutant of SL1344. Attenuated in mice. Well-characterised vaccine strain	[43,45]
SL1344 *atp*	*atpIBEFHAGDC* deletion mutant in SL1344	This study
SL1344 F_0	*atpBEF* deletion mutant in SL1344	This study
SL1344 F_1	*atpHAGDC* deletion mutant in SL1344	This study
SL1344 *atp* (*malXY atp* operon^{+})	SL1344 *atp* complemented by insertion of *atp* operon into the *malXY* pseudogene region	This study
SL1344 *atp* (*malXY* Cm^R)	SL1344 *atp* with chloramphenicol resistance cassette inserted into the *malXY* pseudogene region	This study
pBADλred	Plasmid expressing *exo bet gam* genes from bacteriophage lambda *Exo bet gam* genes as *Nco*I-*Hind*III fragment in pBAD/HisA (invitrogen), Amp^R	[46]
pCP20	Plasmid expressing Flpase genes from *Saccharomyces cerevisiae*. *ClaI-XbaI* fragment of pMMC6 in pHSG415, Amp^R, Cm^R Replicates poorly above 37 °C, stably inherited at 30 °C	[24]
pBADkanFRT	pBADTOPO (Invitrogen, Paisley, UK) containing kanamycin cassette flanked by FRT sites. Kan^R, Amp^R	[46]

(Sigma Dorset UK) supplemented with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 4 μg/ml histidine and the stated carbon source at 0.4% (final w/v).

2.2. Construction of mutants and complementation

Oligo-directed mutagenesis (ODM), an adaptation of ET-cloning, was used to replace the target genes on the *Salmonella* chromosome with a kanamycin resistance cassette flanked with FRT regions from pBADkanFRT [24,25]. PCR was used to amplify the kanamycin resistance FRT cassette with 5′ and 3′ 60 bp arms homologous to DNA flanking the target genes (see Table 2 for primer sequences). *S.* Typhimurium LB5010 containing pBADλred was grown in LB broth supplemented with ampicillin to an OD_{595} of 0.25. Arabinose was added to 0.2% (final w/v) to induce *red* gene expression. Cultures were grown to OD_{595} 0.5 and electroporated with the purified ODM PCR product described above. Mutant colonies were selected on LB agar plates supplemented with 50 μg/ml kanamycin. The desired allelic replacement of the target genes was confirmed by PCR (see Table 2 for primer sequences). Mutations in *S.* Typhimurium LB5010 were transduced into SL1344 by bacteriophage P22 as described previously [26] with selection on LB agar plus kanamycin and gene deletions were confirmed to be correct by PCR and sequencing.

The kanamycin resistance FRT cassette was then excised to leave only a 128 bp FRT scar site. Briefly, electrocompetent mutants of SL1344 were transformed with pCP20 [24] grown at 30 °C and then plated onto LB agar containing 100 μg/ml ampicillin. Single colonies were grown in LB at 39 °C (to prevent replication of pCP20) for 6 h then diluted and plated onto LB agar and incubated overnight at 39 °C. Colonies were screened for loss of ampicillin and kanamycin resistance. Excision of the kanamycin resistance FRT cassette was confirmed by PCR and sequencing to be correct. Southern blot using the FRT scar site region as a probe was also used to confirm that the final mutants were as intended. LPS serotype was confirmed by agglutination with anti-04 serotype antiserum using anti-09 antiserum as a negative control (Remel Europe Ltd./Oxoid Ltd., Basingstoke UK).

For complementation of SL1344 *atp*, lacking the entire *atp* operon, PCR was used to amplify the entire *atp* operon from SL1344 fused to a chloramphenicol resistance cassette, from pACYC184. This was inserted into the *malXY* pseudogene region on the *Salmonella* chromosome using ODM with selection on chloramphenicol. Insertion of the *atp* operon into *malXY* was confirmed by PCR and sequencing of the mutated *malXY* junction and by Southern blotting using *atpG* as the probe. In addition to the complemented strain, SL1344 *atp* (*malXY atp* operon^{+}), a complementation control strain was also generated, SL1344 *atp* (*malXY* Cm^R). For this control strain a chloramphenicol resistance cassette was inserted into the *malXY* pseudogene region of SL1344 *atp* to ensure the insertion into the pseudogene had no phenotypic effects.

2.3. Growth in vitro and succinate utilisation

Cultures in 5 ml of LB broth were incubated overnight with shaking (180 rpm) at 37 °C. Cultures were diluted 1:100,000 into 100 ml of pre-warmed LB broth, and incubated with shaking at 37 °C. Growth was measured by viable count on LB agar plates. Exponential generation times were calculated from growth rates between 4 and 6 h.

To assess the ability to utilise succinate as a sole carbon source wild type and the various *atp* mutants were grown in M9 minimal medium supplemented with 0.4% (w/v) of sodium succinate. Growth was assessed by OD_{595} after 24 and 48 h.

2.4. Mouse typhoid model

Inocula were prepared from overnight cultures grown statically in LB broth at 37 °C. Cultures were centrifuged and bacteria were re-suspended in phosphate buffered saline (pH 7.4) to the required concentration. Seven to nine week-old female BALB/c mice (Harlan, Oxon, UK) were inoculated with 200 μl of bacteria suspension via intravenous injection, or they were lightly anaesthetised with halothane and inoculated by oral gavage. Doses of bacteria given were confirmed by viable counts in LB agar. Gene knock-out mice lacking gp91*phox* or IFNγR1 on a C57/BL6j background where originally purchased from Jackson Laboratory (Bar Marbour, ME) and maintained as homozygous matings at the Wellcome Trust Sanger Institute. C57/BL6j age- and sex-matched control mice were purchased from Harlan (Oxon, UK).

At pre-determined time points postinfection animals were killed, spleens and livers removed and homogenised in 5 ml of sterile water in a Stomacher® 80 Lab System (Seward). Bacterial numbers were enumerated via serial dilutions and plating in LB agar. When required, blood was collected via cardiac puncture under terminal anaesthesia.

For vaccination studies, animals were immunised intravenously with 10^5 CFU or orally with 10^9 CFU. At these doses, immunising strains did not induce clinical signs, were completely cleared with all mice surviving the infection. At 13 weeks postimmunisation clearance of the bacteria was confirmed by viable counts from spleens and livers. Mice were subsequently re-challenged either