JOURNAL OF BACTERIOLOGY, June 1997, p. 4046–4048 Vol. 179, No. 12
0021-9193/97/$04.00+0

Identification of the *ahp* Operon of *Salmonella typhimurium* as a Macrophage-Induced Locus

KEVIN P. FRANCIS,† PATRICK D. TAYLOR, CHRISTOPHER J. INCHLEY, AND MAURICE P. GALLAGHER*

Division of Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland

Received 31 December 1996/Accepted 16 April 1997

Previously, we tagged a macrophage-induced *Salmonella typhimurium* locus with Mu*dlux* (K. P. Francis and M. P. Gallagher, Infect. Immun. 61:640–649, 1993). The insertion lies within the OxyR-regulated *ahpC* locus and conveys alkyl peroxide sensitivity. Plasmid-encoded *ahp* reverses sensitivity but reduces luminescence. This suggests that OxyR is titrated by the multicopy *ahp* promoter.

Salmonella typhimurium is an enteroinvasive pathogen of mice and results in a typhoid-like disease. Following oral ingestion, *S. typhimurium* cells are thought to invade and traverse the epithelial lining of the intestine (7), spreading to the surrounding tissues, where they are engulfed by macrophages. Generally, virulence loci of *S. typhimurium* have been identified by screening transposon mutants for alterations in host cell invasion, intracellular survival, or intracellular multiplication (6, 7, 9), and for the most part, such mutants have correlated well with attenuation.

Exposure of *S. typhimurium* to hydrogen peroxide results in a multigenic response, with selective induction of approximately 30 polypeptides (4, 12), of which a subset of 9 are regulated by the transcriptional activator OxyR. Recently, we reported the construction of a pool of Mu*dlux* fusions in the virulent strain *S. typhimurium* SL1344 (8). One of the isolates from this pool, MPG203, was found to luminesce not only in direct response to exposure to hydrogen peroxide but also during infection of cells of the murine macrophage line J774.2.

A number of OxyR-regulated proteins have been characterized (1, 4). Two of these, in particular, catalase (encoded by *katG*) and alkyl hydroperoxide reductase (encoded by *ahp*), can be distinguished by the ability to convey resistance to hydrogen peroxide or alkyl peroxides (such as cumene peroxide), respectively. When a lawn of MPG203 cells was examined for such properties, they exhibited enhanced sensitivity to cumene in comparison to the parental cell type, SL1344 (Table 1), but not to hydrogen peroxide. This strongly suggested that the Mu*dlux* element had inserted itself within the *ahp* locus. We attempted to confirm this by PCR, using oligonucleotides which corresponded to the 5′ end of the sense strand of the coding sequence of the first gene of the *ahp* operon, *ahpC* (bases 150 to 167 [17]), and to a 20-base region in the terminus of the β segment of phage Mu (bases 83 to 64 [11]). A fragment of approximately 400 bp was amplified and cloned into the *Eco*RI and *Sma*I sites of pBluescript SK$^+$ following blunt ending and *Eco*RI digestion. (This made use of the natural *Eco*RI site close to the start of the *ahpC* coding region [17]). Sequence analysis revealed that the Mu*dlux* element had inserted itself 318 bases downstream from the translational start of *ahpC* (data not shown).

The *ahp* locus is known to be regulated by the transcriptional activator OxyR. To confirm that the Mu*dlux* gene fusion was correctly regulated, it was transduced, with P22, into LT2 derivatives TA4100 and TA4108 (4), in which *oxyR* is either expressed constitutively or has been deleted, respectively. The responses of exponentially growing cultures of the resulting strains (designated MPG351 and MPG352) to hydrogen peroxide were then assessed in comparison to MPG203 and an LT2 derivative carrying the *ahp*::Mu*dlux* fusion (designated MPG350). MPG351 exhibited extensive light production throughout the experiment (Fig. 1), even in the absence of hydrogen peroxide, while MPG352 produced no significant light under any conditions. A typical response was observed from MPG203 and MPG350 (8). These observations firmly

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Relevant properties	Source or reference
SL1344	*his*; virulent mouse pathogen	10
MPG203	*his ahpC*::Mu*dlux* (Km^r); SL1344 derivative	8
TA4100	*oxyR*^{c}; LT2 derivative	4
TA4108	Δ*oxyR*; LT2 derivative	4
MPG350	*ahpC*::Mu*dlux* (Km^r); LT2 derivative	This study
MPG351	*oxyR*^{c} *ahpC*::Mu*dlux* (Km^r); TA4100 derivative	This study
MPG352	Δ*oxyR ahpC*::Mu*dlux* (Km^r); TA4108 derivative	This study
pBR322	Tc^r Ap^r	2
pBluescript SK$^+$	*lacZ*α Ap^r	14
pACYC184	Cm^r	3
pDSA23	pACYC184 derivative carrying *ahpCF* locus from *E. coli*; Ap^r	15
pPDT12[b]	pBR322 carrying *S. typhimurium ahp* region; both *ahpC* and *ahpF* disrupted by insertion of *cml* cartridge	This study

[a] Strains were derived from *S. typhimurium* LT2 or SL1344. P22 *HT int4* (13) was used for transduction.

[b] pPDT12 was constructed by amplification of *ahpCF* from within the *orfO* promoter of *S. typhimurium* (16) to a region at the 3′ end of *ahpF* (accession no. J05478; base 2508) by using primers 1 and 2, containing *Bgl*II and *Hin*dIII sites added to the 5′ ends, respectively (see below). The product was cloned into the *Bam*HI and *Hin*dIII sites of pBR322, and the *cml* gene of pBR325 (bases 4288 to 5270; accession no. L08855) was then amplified (by using primers 3 and 4, which contain a 5′ *Hpa*I or *Mlu*I site, respectively) and inserted into the corresponding natural sites of *ahpCF*. Primers 1 to 4 (with restriction sites underlined) were as follows: 1, 5′-CCAGATCTGCCTATCACAGACATAGG-3′; 2, 5′-CGAAGCTTGGTGCGAATCAGATAAT-3′; 3, 5′-GGGATATCAAGATTGTTAACCGTCTAAGAAACCATTATTATCATG-3′; 4, 5′-GCGATATCAAGCTTACGCGTCGTAGCACCAGGCGTTTAAGGGCAC-3′.

* Corresponding author. Phone: (0131) 650 5409. Fax: (0131) 668 3870. E-mail: mp.gallagher@ed.ac.uk.

† Present address: ABFS (Microbiology), Sutton Bonnington CAMPL, North Loughborough, Leicestershire LE12 5RD, England.

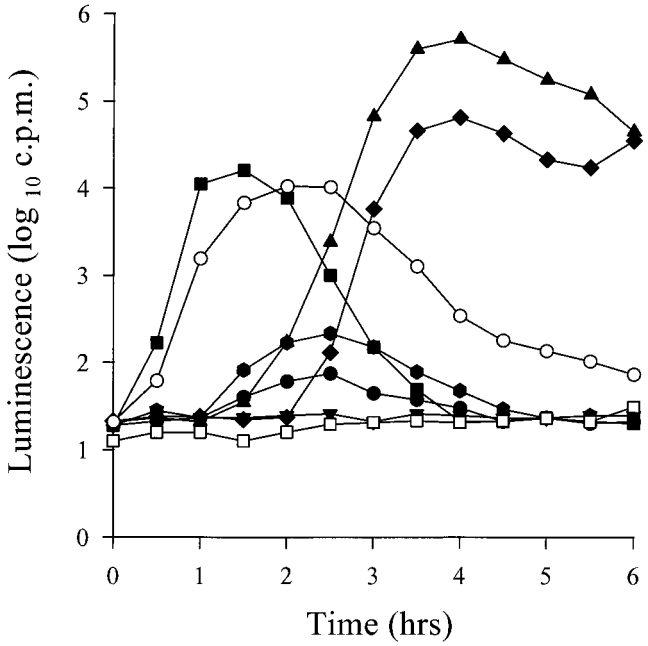

FIG. 1. The Mu*dlux* fusion in MPG203 is subject to regulation by OxyR. Cultures, grown overnight in Luria-Bertani medium containing kanamycin (50 μg ml^{-1}) where appropriate, were diluted into fresh medium to approximately 10^4 CFU ml^{-1} and then incubated aerobically for 90 min at 30°C. Subsequently (time zero), bioluminescence was determined in a liquid scintillation counter (Beckman LS1701) by using the tritium channel in the absence (MPG203, inverted triangles; MPG351, diamonds; MPG352, hexagons; MPG350, open squares) or presence (MPG203, squares; MPG351, triangles; MPG352, circles; MPG350, open circles) of added hydrogen peroxide (20 μM).

establish that OxyR is involved in the transcriptional regulation of the target gene containing the Mu*dlux* element.

Introduction of plasmid pDSA23, which carries the intact *ahp* locus from *Escherichia coli* on pACYC184 (15), into MPG203 or SL1344 resulted in a significant reduction in the zone of inhibition caused by cumene and an increase in the zone of inhibition caused by hydrogen peroxide (Table 2). This finding is in agreement with previous studies with *E. coli* (17). Moreover, from Fig. 2a, it can also be seen that pDSA23 resulted in the virtual abolition of hydrogen peroxide-induced luminescence from MPG203, although a typical pattern of light expression was observed from MPG203 containing the parental plasmid pACYC184. To determine whether this reflected a detrimental effect of pDSA23 on MPG203 through high-level *ahpCF* expression or, alternatively, reflected the indirect effects of OxyR sequestration by the plasmid, the experiments were repeated with MPG203 cells containing pPDT12. This plasmid carries the *ahp* operon (including the promoter region) of *S. typhimurium* cloned into pBR322. However, in pPDT12, the *ahpCF* genes have been disrupted by insertion of the PCR-amplified *cml* locus from pBR325 into the *Mlu*I and *Hpa*I sites of *ahpC* and *ahpF*, respectively (Table 1). When MPG203(pPDT12) was exposed to hydrogen peroxide (Fig. 2b), its luminescence was reduced, as previously found with pDSA23, suggesting that the reduced light production arose as a consequence of OxyR titration. It is likely that this also explains the enhanced sensitivity of MPG203(pDSA23) and SL1344(pDSA23) to hydrogen peroxide observed on solid medium (Table 2).

In conclusion, we have provided clear evidence that the Mu*dlux* element in MPG203 lies within the *ahpC* gene of *S. typhimurium* and is regulated by OxyR. This directly implicates the *ahp* locus in responding to the oxidative burst of macrophages and highlights the potential of AhpCF and, possibly, other OxyR-regulated gene products as in vivo-expressed an-

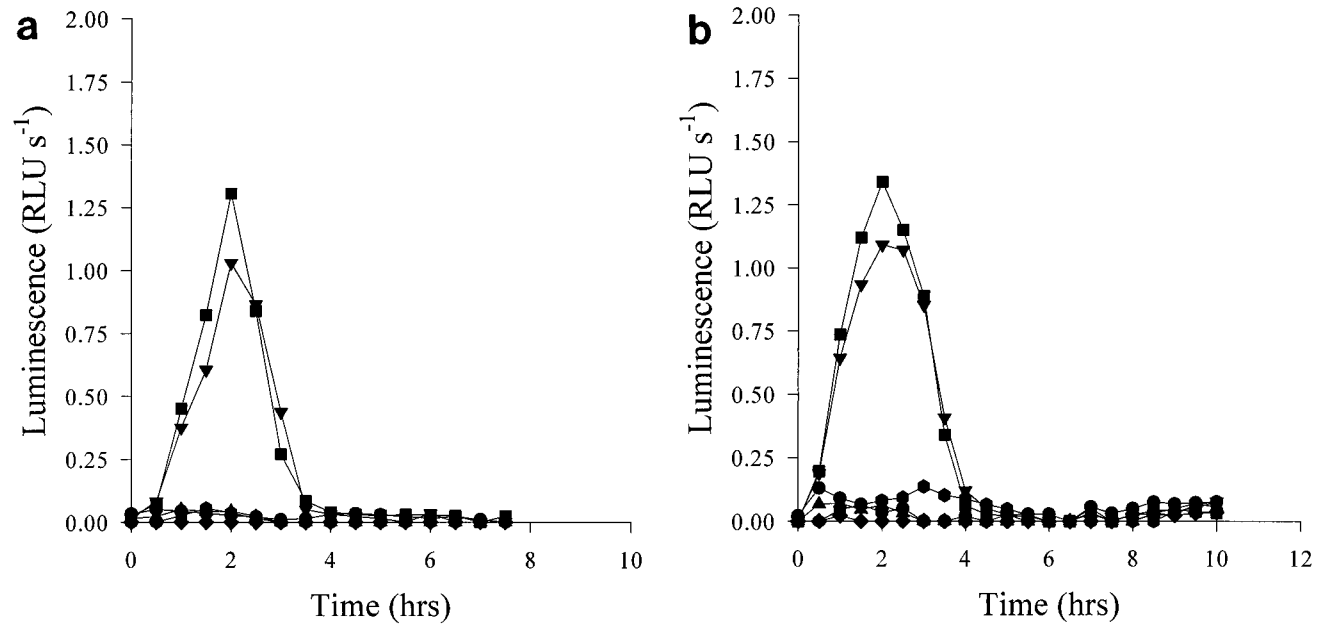

FIG. 2. Effects of plasmid-mediated expression of *ahp* on bioluminescence from MPG203. Cultures, grown overnight in Luria-Bertani medium containing ampicillin or chloramphenicol (50 μg ml^{-1}), were diluted into fresh medium to approximately 10^4 CFU ml^{-1} and then incubated aerobically for 90 min at 30°C. Subsequently (time zero), bioluminescence was determined with a Luminoskan RS 96-well plate luminometer (Life Sciences Int.) at 30°C in the absence [a—MPG203, circles; MPG203(pDSA23), diamonds; MPG203(pACYC184), triangles; b—MPG203, circles, MPG203(pPDT12), diamonds; MPG203(pBR322), triangles] or presence [a—MPG203, squares; MPG203(pDSA23), hexagons; MPG203(pACYC184), inverted triangles; b—MPG203, squares; MPG203(pPDT12), hexagons; MPG203(pBR322), inverted triangles] of hydrogen peroxide (3% [vol/vol]). RLU, relative light units.

TABLE 2. Sensitivities of *S. typhimurium* strains to peroxides[a]

Strain (genotype)[b]	Zone of inhibition (dia, mm)	
	Cumene peroxide	Hydrogen peroxide
SL1344	22	16
MPG203 (*ahp*)	36	18
LT2	22	16
TA4108 (Δ*oxyR*)	31	30
TA4100 (*oxyR*^{c})	15	10
MPG203 (*ahp*/pDSA23 *ahpCF*$^+$)	16	25
MPG203 (*ahp*/pACYC184)	36	18
MPG203 (*ahp*/pPDT12 *ahpC′F′*::*cml*)	36	24
MPG203 (*ahp*/pBR322)	36	18
SL1344(pDSA23) (*ahpCF*$^+$)	15	24
SL1344(pACYC184)	22	16
SL1344(pPDT12) (*ahpC′F′*::*cml*)	27	24
SL1344(pBR322)	22	17

[a] Cells were grown overnight in Luria-Bertani medium containing kanamycin, ampicillin, or chloramphenicol (each at 50 μg ml^{-1}) when appropriate. A 0.1-ml sample was spread on Luria-Bertani agar, and 10 μl of a 3% solution of cumene peroxide or hydrogen peroxide was added to a cellulose nitrate antibiotic assay disc (6-mm diameter) placed in the center of the plate. The plates were incubated at 30°C overnight, and the diameters of the zones of inhibition were determined. Measurements are averages of duplicate tests. Duplicates differed by less than 1 mm in all cases.

[b] Only relevant genotypic properties are shown.

tigens. In relation to this, it is also noteworthy that expression of *ahpC* by *Mycobacterium* species has been reported during their interaction with macrophages (5). Sequestration of OxyR by the *ahp* promoter carried on a multicopy plasmid may also have implications for the use of this promoter for in vivo expression of foreign antigens by using live attenuated *Salmonella* strains as vehicles for immunization.

Our thanks to D. Smillie and R. Hayward for helpful discussions.

This study was supported by the Nuffield Foundation, the Royal Society, and the B.B.S.R.C. K.P.F. and P.D.T. were recipients of B.B.S.R.C. studentships.

REFERENCES

1. **Altuvia, S., M. Almiron, G. Huisman, R. Kolter, and G. Storz.** 1994. The *dps* promoter is activated by OxyR during growth and by IHF and a sigma(s) in stationary-phase. Mol. Microbiol. **13:**265–272.
2. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, and H. W. Boyer.** 1977. Construction and characterisation of a new cloning vehicle. II. A multipurpose cloning system. Gene **2:**95–113.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the pA15 cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
4. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defence against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753–762.
5. **Dhandayuthapani, S., L. E. Via, C. A. Thomas, P. M. Horowitz, D. Deretic, and V. Deretic.** 1994. Green fluorescent protein as a marker for gene-expression and cell biology of mycobacterial interactions with macrophages. Mol. Microbiol. **17:**901–912.
6. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
7. **Finlay, B. B., M. N. Starnbach, C. L. Francis, B. A. D. Stocker, S. Chatfield, G. Dougan, and S. Falkow.** 1988. Identification and characterisation of Tn*phoA* mutants of *Salmonella* that are unable to pass through a polarized MDCK epithelial cell monolayer. Mol. Microbiol. **2:**757–766.
8. **Francis, K. P., and M. P. Gallagher.** 1993. Light emission from a Mu*dlux* transcriptional fusion in *Salmonella typhimurium* is stimulated by hydrogen peroxide and by interaction with the mouse macrophage cell line J774.2. Infect. Immun. **61:**640–649.
9. **Gahring, L. C., F. Heffron, B. B. Finlay, and S. Falkow.** 1990. Invasion and replication of *Salmonella typhimurium* in animal cells. Infect. Immun. **58:** 443–448.
10. **Hosieth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.
11. **Kahmann, R., and D. Kamp.** 1979. Nucleotide sequence of the attachment sites of the bacteriophage Mu DNA. Nature **280:**247–250.
12. **Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. N. Ames.** 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. USA **83:**8059–8063.
13. **Roth, J.** 1970. Genetic techniques in the studies of bacterial metabolism. Methods Enzymol. **17A:**3–35.
14. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. Lambda ZAP: a bacteriophage lambda expression vector with *in vivo* excision properties. Nucleic Acids Res. **16:**7583–7600.
15. **Smillie, D. A., R. S. Hayward, T. Suzuki, N. Fujita, and A. Ishihama.** 1992. Location of the genes encoding alkyl hydroperoxide reductase on the physical map of the *Escherichia coli* K-12 genome. J. Bacteriol. **174:**3826–3827.
16. **Tartaglia, L. A., C. J. Gimeno, G. Storz, and B. N. Ames.** 1992. Multidegenerate DNA recognition by the OxyR transcriptional regulator. J. Biol. Chem. **267:**2038–2045.
17. **Tartaglia, L. A., G. Storz, M. H. Brodsky, A. Lai, and B. N. Ames.** 1990. Alkyl hydroperoxide reductase from *Salmonella typhimurium*: sequence and homology to thioredoxin reductase and other flavoprotein disulphide oxidoreductases. J. Biol. Chem. **265:**10535–10540.