

Neutrophil Microbicidal Activity: Screening Bacterial Mutants for Survival after Phagocytosis Using Quantitative PCR

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SUMMARY: When a constant gene replacement sequence is introduced into bacteria to produce mutants and the flanking chromosomal sequences are known, it is possible to use a quantitative polymerase chain reaction method (QPCR) to compare the concurrent survival of the different bacterial mutants under identical conditions. We describe *Escherichia coli* survival following neutrophil phagocytosis among three mutants deleted respectively for *araB*, *dps* or *oxyR*. Comparisons were made both by traditional and QPCR methods with similar results and indicate that the survival defect of an *oxyR* and *oxyS* mutant described previously can be attributed to the loss of *oxyR* alone. Deletion of *dps*, a prominent member of the regulon controlled by the *oxyR* gene product does not engender a survival defect. We suggest that QPCR analysis can readily compare the relative survival of 10 or more mutants concurrently. QPCR analysis would seem to be especially valuable when experimental conditions are subject to a high degree of sample to sample variability or when the stress producing system involves use of expensive or scarce resources like rare patient cells, cells from children, or the use of genetically modified animal hosts.

Expression profiling of *Escherichia coli* phagocytosed by human neutrophils identified a number of genes with increased expression in response to stresses generated in the phagosome (1). We made replacement mutations for a number of these genes (2) and sought to develop an efficient method for determining the phenotype of these mutations with respect to survival after neutrophil phagocytosis using the quantitative polymerase chain reaction (QPCR) to estimate concurrent survival of different mutants confronting the same microbicidal conditions. This report describes aspects of these efforts.

METHODS

E. coli strain EC1 is ATCC 11775 (American Type Culture Collection). Plasmid pKD46 encodes λ -phage recombinase enzymes (2). EC1 was transformed with pKD46 to give EC218.

Previously described procedures: The gene replacement procedure was as described by Datsenko and Wanner (2) with modifications (available from the authors) to address the fact that EC1 and EC218 are heavily encapsulated and metabolize arabinose actively. Human neutrophils and serum were prepared as previously described (1). The traditional bacterial survival assay (1) was modified with respect to bacterial and neutrophil cell numbers as described in the text and Figure legends.

Competitive survival after neutrophil phagocytosis: Bacterial colonies from Mueller-Hinton agar plates prepared during viability assays of mixed strains (300 - 1,000 colonies per plate) were collected by repeated swabbing (sterile cotton-tipped swab) and rinsing in 1 mL of TE. Genomic DNA was prepared from 0.3 mL of TE suspension by a method adapted from (3) as further modified in <http://research.umbc.edu/~jwolf/ml.htm> and used as a template for quantitative PCR as described below.

Quantitative PCR: 10 μ L of 2x master mix containing Sybr Green (SYBR Green PCR Master Mix, Applied Biosystems) were combined with 10 ng mixed *E. coli* template DNA and 25 pmol of each appropriate primer in a final volume of 20 μ L. Cycling and detection was on a Rotor-Gene 2000 (Corbett Research): 10' at 95°C to activate the polymerase followed by 50 cycles alternating between a 95°C denaturation step (10 s) and a 60°C annealing-extension step (30 s). Fluorescence data was acquired on the FAM channel during the 60°C step, with a gain setting of 4.

RESULTS

Mutants: Mutant strains of the uropathogen, EC1, were constructed as described in Methods. Targeted genes were those for *oxyR*, *dps*, a prominent neutrophil-stress regulated gene of the OxyR-regulon, and *araB*. The goal for constructing the *araB* strain was to use it as a control strain containing a PCR detectable chromosome modification that would not affect the response to neutrophil-

mediated stress.

Traditional bacterial survival assay: When survival of these strains was evaluated in a typical neutrophil microbicidal assay (Fig. 1), both the *araB* and *dps* strains were killed at rates comparable to the wild type parent, whereas killing of the *oxyR* strain was considerably more extensive. Thus the *araB* mutation did not alter bacterial survival in response to neutrophil microbicidal systems and could be used as a wild-type surrogate in further experiments. Interestingly, although the *dps* mutation is known to confer a measure of hypersusceptibility to reagent hydrogen peroxide in *E. coli* (4) (confirmed by us in our mutant) and is a virulence factor in salmonella pathogenesis models (5), it bears no detectable phenotype in the neutrophil killing assay. Also, it appears that the *oxyR* mutation has the same phenotype as the *oxyRS* mutation evaluated previously (1) and thus *oxyS* appears to be unnecessary for the manifestation of the neutrophil hypersusceptibility phenotype.

Bacterial survival assays for mixed strains: Overnight cultures

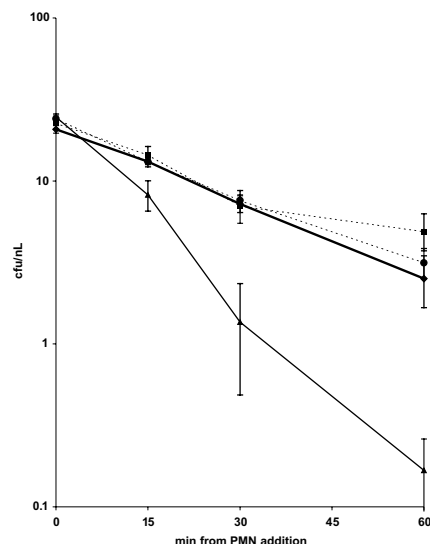


Fig. 1. Bacterial survival after neutrophil phagocytosis - traditional method.

Bacteria (20×10^6) were pre-opsonized with 5.5% fresh human serum for 30 min and incubated with 5×10^6 neutrophils. At indicated intervals samples were taken to assess residual bacterial viability. Bacterial strains were wild type EC1 ($n = 8$, heavy solid line, diamond symbols), and gene replacement mutants *araB* ($n = 5$, dashed line, circles), *dps* ($n = 4$, dashed line, squares), and *oxyR* ($n = 9$, thin solid line, diamonds). Error bars reflect standard error estimates from the log transformed data.

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of different strains were combined (10% *araB*, 10% *dps*, 10% *oxyR* and 60% wild-type), washed, and samples were plated to obtain individual colonies prior to the addition of serum and neutrophils (INPUT SAMPLE). After the addition of serum and neutrophils, further samples were plated to enumerate survivors (OUTPUT SAMPLE). Overall survival of the bacterial mixture in response to neutrophil phagocytosis was assessed by colony counting. The initial inoculum of 20 ± 2 million bacteria per mL declined to 0.2 ± 0.1 (mean \pm SD, triplicate samples, 1 experiment) during the 90 min incubation: 30 min with serum and an additional 60 min with neutrophils. Total DNA was prepared from colonies swabbed from INPUT and OUTPUT plates as described in the methods section. The relative abundance of each mutant in the INPUT and OUTPUT samples was assessed by QPCR.

QPCR: Fig. 2 describes the features of INPUT and OUTPUT DNA samples (triplicate killing assays performed on the same day, resulting in 3 INPUT and 3 OUTPUT samples) analyzed, using the appropriate primers and Sybr Green fluorescence, to determine the proportionate abundance of the *araB*, *dps*, and *oxyR* genotypes in the sample. The relative abundance of the *araB* gene disruption sequence was highly reproducible among the 3 INPUT samples (solid lines) and the slight shift to the left for the OUTPUT strains (dotted lines) suggests a slight increase of the representation of the *araB* gene disruption sequence in these samples. This shift can be quantified by establishing a threshold value and determining the number of PCR cycles required to achieve this level of fluorescence (C_t = cycles to threshold). For the INPUT samples, with a threshold of 0.01 fluorescence units, the *araB* C_t was 18.8 ± 0.3 (mean \pm SEM, $n = 3$). For the OUTPUT samples C_t was 17.8 ± 0.2 , a difference of 1.0 cycles, $P < 0.02$.

For the *dps* mutation, the increased representation in the OUTPUT sample was less dramatic (INPUT $C_t = 18.7 \pm 0.1$, OUTPUT $C_t = 18.1 \pm 0.2$) but still significant, $P < 0.05$. The increased representation of the *araB* and *dps* strains in the OUTPUT samples may be accounted for, in part, by compensation for the decline in the representation of the *oxyR* strain. The C_t for the *oxyR* strain in the INPUT samples averaged 18.5 ± 0.1 , while the OUTPUT samples averaged 21.2 ± 0.1 , a shift of nearly 3 cycles, $P < 0.005$. Assuming that each cycle reflects a 2-fold difference in template abundance (100% PCR efficiency), the C_t differences among the strains suggests a 7-15 fold decrease in the survival of the *oxyR* strain compared to the *araB* and *dps* strains. The result is quite consistent with the viable results described in Figure 1 and indicates that QPCR screening can be effective at detecting strains with altered survival compared to their peers in a competitive survival assay.

DISCUSSION

Constructing gene deletions by the facilitated homologous recombination method described by Datsenko and Wanner (2) inserts a known replacement DNA sequence at a precise location in the

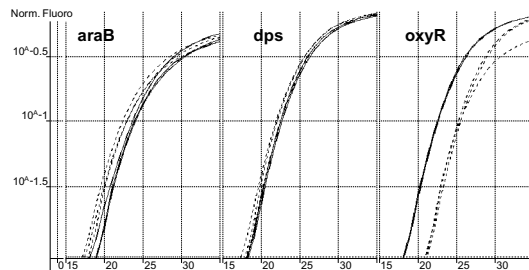


Fig. 2. QPCR of DNA from colonies arising from mixed suspensions of *E. coli*. Sybr Green method.

Three INPUT samples containing DNA from a mix of wild type (60%), *araB* (10%), *dps* (10%) and *oxyR* (10%) gene replacement mutants were evaluated for relative abundance of the replaced DNA sequences using appropriate primer sets (Table 1) and 10 ng of total DNA from the three separate samples (solid line amplification plots). After 30 min opsonization with serum and 60 min incubation with neutrophils, as described in Fig. 1, OUTPUT DNA was obtained, as described in methods (dashed line amplification plots).

chromosome. When combined with knowledge of the flanking chromosomal sequence, it is possible to define a region of DNA sequence, part chromosomal and part insert, that is unique to the newly constructed mutant strain and that distinguishes it from all other mutants constructed by this method. By locating one PCR primer in the insert and a corresponding primer in the chromosome, it should be possible to amplify that unique sequence in a QPCR reaction.

The number of PCR cycles required to reach an arbitrary threshold of detection, typically correlates with the number of copies of DNA template introduced into the QPCR reaction. The mutations evaluated in this work are those for *araB*, *oxyR* and *dps*. The *araB* gene is the first in the *araBAD* operon that contributes to arabinose metabolism. There is no known relationship between arabinose metabolism and bacterial interaction with neutrophil microbicidal systems. Indeed neutrophils killed the *araB* mutant at the same rate as the wild type parent (Fig. 1).

The *oxyR* gene encodes a transcription factor that is relatively quiescent in its reduced form but becomes active following a conformational change associated with a more oxidizing cytosolic environment. A gene replacement mutant that eliminated both *oxyR* and *oxyS*, was observed to be hypersusceptible to neutrophil-mediated killing systems (1). In the current study, we have shown that deletion of just *oxyR* is sufficient to produce this phenotype (Fig. 1).

Among the bacterial mRNA's that increase dramatically in abundance during neutrophil phagocytosis, is that for the *dps* protein. The *dps* protein binds to DNA, sequesters iron in the bacterial cytosol in a ferritin-like manner (6,7), and confers substantial

Table 1. PCR primers and probes

oligonucleotide name	Sequence	
λ-red gene disruption	sense	anti-sense
pKD3-homology	<i>GTGTAGGCTGGAGCTGCTTC</i>	<i>CATATGAATATCCTCCTTAG</i>
L-RED-araB-f	TTATAGAGTCGCAACGGCCTGGCAGCCTGTGCCGGGGC ¹	ATGGCGATTGCAATTGGCCTCGATTTTGGCAGTGATTCT ¹
L-RED-dps-f	ATGAGTACCCTAAATTAGTTAAATCAAAGCGACCAAT ¹	TTATTTCGATGTTAGACTCGATAAACCCACAGGAATTTATC ¹
L-RED-oxyR-f	ATGAATATTCGTGATCTTGAGTACCTGGTGGCATTGGCTG ¹	TTAATGCACGGCAGATAAACACCCCATCGCGTTTGGCT ¹
sybr Green QPCR²		
araB-sybr	<i>ATTATTATTCTGCGAAGTGATCTTCCGTCACA</i>	<i>GCGATTGCAATTGGCCTCGATTTTG</i>
dps-sybr	GAGTACCCTAAATTAGTTAAATCAAAGCGACC	<i>GCGCGCCATTAAATGAAGTTCCTATTCCG</i>
oxyR-sybr	AATATTCGTGATCTTGAGTACCTGGTGGCA	<i>GCGCGCCATTAAATGAAGTTCCTATTCCG</i>

¹: Sequence extended by the sequence indicated by "pKD3-homology" in the first line.

²: Primers for detection of λ -red recombinants using sybr-Green fluorescence. Sequences that recognize the insertion element are in large italic font.

resistance to hydrogen peroxide-mediated toxicity (4). However deletion/replacement of the *dps* gene did not affect EC1 survival in neutrophils (Fig. 1).

When the three mutant strains (10% each) were mixed with wild-type and incubated with neutrophils for 60 min, the overall mixture experienced a 99% decline in viability. Among the survivors, the *araB* and *dps* genotypes were slightly over-represented while the *oxyR* genotype was significantly under-represented as determined by QPCR analysis (Fig. 2). The QPCR analysis achieved the same result in a single experiment with three replicate samples as was observed in 4-9 experiments employing traditional methods. Based on the 10% abundance of each mutant in the INPUT mixture, we estimate that QPCR analysis could easily detect differences within a mixture of 10 different strains and perhaps many more. We would suggest that QPCR analysis could serve as an effective screening method to select promising candidates for further study from among many mutants.

Preliminary studies have indicated that single gene mutations are insufficient to render EC1 hypersusceptible to neutrophil microbicidal systems but that double and triple mutations acquire a significant phenotype (unpublished). We propose to use QPCR analysis to further screen additional mutants of EC1, examining the contribution of key genes of the *oxyR* regulon individually and in combination.

ACKNOWLEDGMENTS

This work was supported by a grant AI049417 from the National Institutes of Health.

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