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Stress-inducible gene of *Salmonella typhimurium* identified by arbitrarily primed PCR of RNA

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ABSTRACT **Fingerprinting of RNA by arbitrarily primed PCR (RAP) can be used to identify conditionally expressed genes in prokaryotes. Differential gene expression in *Salmonella typhimurium* LT2 in response to peroxide treatment was examined as a system in which to demonstrate this strategy. This treatment models the induction of bacterial protective proteins that may occur when mammalian phagocytes use peroxide to fight *S. typhimurium* infection. To identify genes inducible by hydrogen peroxide stress, total RNA from peroxide-treated and untreated bacterial cultures were RAP fingerprinted with six different arbitrarily selected primers. A 435-base RAP product that was differentially amplified by RAP using the reverse sequencing primer was cloned and sequenced. Northern blot analysis confirmed that the RNA corresponding to this clone, RSP435, was induced when bacteria were treated with hydrogen peroxide. The RNA was not induced in an *oxyR1* mutant that constitutively expresses a subset of hydrogen peroxide-inducible genes. Using pulsed-field gel electrophoresis and dot blot hybridization to an array of induced Mu*d*-P22 integrations, the gene corresponding to RSP435 was mapped to two places, one between 19 and 21.5 min and one between 56 and 57 min. Thus, two similar or identical stress-inducible genes were found in different parts of the genome. Identification, cloning, and mapping of the conditionally expressed RSP435 cDNA were performed entirely by physical means, demonstrating that the strategy should complement genetic methods for many prokaryotic or archaebacterial systems and should be applicable to organisms in which genetic methods are difficult to perform or have not yet been developed.**

Two similar methods for fingerprinting of eukaryotic RNA have been developed (1, 2) based on arbitrarily primed PCR (RAP) (3, 4). In principle, fingerprinting of RNA by RAP (1, 5) can be used on prokaryotic RNAs because it does not rely on poly(A)-tailed RNA that is rare in mRNAs isolated from bacteria (6). To demonstrate that the method can be used to identify differentially expressed genes of bacteria we searched for stress-induced RNAs in *Salmonella typhimurium*.

Enterobacteria respond to various stimuli such as oxidative stress, extreme pH, anaerobiosis, heat shock, osmotic shock, and starvation, by changing the expression of groups of genes termed "stimulons" (7, 29). Assorted genetic tools have been used to study these regulatory networks, including operon fusions (8), promoter probe plasmids (9, 10), and positively selected promoter probes (11). An example of the differential expression of sets of genes due to external stimuli is the response of *S. typhimurium* to oxidative stress. When bacteria invade the mammalian host, activated granulocytes may employ millimolar concentrations of peroxide and superoxide anion during phagocytosis in an attempt to kill the bacteria (12, 13). In response, the bacteria can induce stimulons, including those under the control of *oxyR* (12). Although the exact roles of genes induced by peroxide stress are unknown, the induction of some of these genes is required to maintain virulence. In this report, we describe the application of RAP to identify genes in *S. typhimurium* that are induced by H_2O_2 stress.

MATERIALS AND METHODS

Strains. Strains used are listed in Table 1. We thank the following for providing strains: K. E. Sanderson (University of Calgary, AB Canada), J. R. Roth (University of Utah, Salt Lake City), G. Stortz (National Institute of Child Health and Human Development, Bethesda, MD), and A. Anderson (L'Hotel-Dieu de Quebec). All strains are *S. typhimurium* LT2 derivatives. A complete list of Mu*d*-P22 strains has been published (14, 15).

Peroxide-Induced Stress. *S. typhimurium* LT2 (wild type) and the LT2 derivative strain TA4100 (*oxyR1*) (12) were grown aerobically as described (19). A 1% inoculum of overnight aerobic culture was grown to logarithmic phase (OD_{650} = 0.4) in L broth. The culture was immediately split into two aliquots and H_2O_2 was added to one of the aliquots to a final concentration of 0.38 mM. This level of H_2O_2 is less than that needed to stop the growth of the cells, is a fraction of the level that the cells are thought to experience during phagocytosis (13), yet is sufficient to prepare cells for survival in 10 mM H_2O_2, which would otherwise be lethal. After H_2O_2 was added, the cultures were incubated aerobically at 37°C for either 30 min or 120 min. At each time interval, 10 ml of the culture was removed and frozen immediately in a dry ice/ethanol bath.

RNA Preparation. RNA was isolated by the hot phenol extraction procedure (20). The 10 ml of frozen cell culture was thawed and harvested by centrifugation in a 15-ml Corex tube at 10,000 rpm for 10 min. Cell pellets were resuspended in 0.5 ml of 30 mM NaOAc (pH 5.2). Fifty microliters of 20% SDS and 0.5 ml of phenol (equilibrated with 30 mM NaOAc to pH 5.2) were added. The mixture was spun in a Vortex for a few seconds to form an emulsion and placed in a 65°C water bath for 10 min (with Vortex mixing at 2.5-min intervals). The aqueous phase was then separated from the phenol phase by microcentrifugation for 5 min. The aqueous phase was extracted with phenol at 65°C and then once with chloroform at room temperature. RNA was precipitated with 1/4 vol of 3 M NaOAc and 3 vol of ethanol. The RNA was then treated with RNase-free DNase I or pelleted in a CsCl gradient (21). Total RNA was dissolved in water and the different RNA preparations were adjusted to equal concentrations. The relative concentrations of these final RNA stocks were compared by ethidium gel electrophoresis.

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Abbreviations: RAP, RNA fingerprinting by arbitrarily primed PCR; PFGE, pulsed-field gel electrophoresis; RSP, reverse sequencing primer.

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Table 1. Strains used in this study

Strain	Relevant genotype	Source	Ref.
LT2	Wild type	K. E. Sanderson	28
TT3338	*gal*::Tn*10*	J. R. Roth	28
TA4100	*oxyR1*	G. Storz	12
TAQ100	*Fels*-1$^-$ *Fels*-2$^-$	A. Anderson	23
TAQ100F1	*Fels*-1$^+$ *Fels*-2$^-$	A. Anderson	23
TAQ100F2	*Fels*-1$^-$ *Fels*-2$^+$	A. Anderson	23
KKWQ100	*gal*::Tn*10**	TAQ100 × P22(TT3338)	
KKWQ100F1	*gal*::Tn*10**	TAQ100F1 × P22(TT3338)	
KKWQ100F2	*gal*::Tn*10**	TAQ100F2 × P22(TT3338)	

Fifty-two strains, each containing a Mu*d*-P22 prophage insertion placed at about 5-min intervals around the *S. typhimurium* LT2 genome, are not listed. When the prophage is induced by mitomycin C, the phage will pack about three headful of chromosomal DNA from one direction depending on the *pac* site within the prophage (14, 15).

*Constructed by generalized transduction with P22 HT12/4 *int3* (16) grown from the donor strain TT3338 (*gal*::Tn*10*). As a result, a *Bln* I fragment of about 200 kb, which corresponds to 18–21.5 min, was "dropped out" from the *Bln* I-A fragment (1600 kb) when the chromosome was digested with *Bln* I and resolved by pulsed-field gel electrophoresis (PFGE) (17, 18).

Fingerprinting of RNA. RAP was employed essentially as described by Welsh *et al.* (1). Ten-microliter samples containing 3 ng, 12 ng, 50 ng, or 200 ng of RNA were incubated at 65°C for 10 min and then put on ice. Ten microliters of cDNA synthesis buffer containing 100 mM Tris·HCl (pH 8.3), 100 mM KCl, 8 mM $MgCl_2$, 20 mM dithiothreitol, 2 mM dNTPs, 1 μM arbitrarily chosen primer, and 5 units of Moloney murine reverse transcriptase (Stratagene) was added. The first strand cDNA synthesis was carried out at 37°C for 1 hr. After cDNA synthesis, 10 μl of buffer containing 2 mM Tris·HCl (pH 8.3), 5 mM KCl, 4 mM $MgCl_2$, 1 μM of the same arbitrarily chosen primer, 1 μCi of [α-^{32}P]dCTP (1 Ci = 37 GBq), and 0.25 unit of Taq polymerase (AmpliTaq, Cetus) was added to the 20-μl cDNA sample. The thermal cycling parameters were as follows: 1 low stringency cycle; 94°C (5 min), 40°C (5 min), 72°C (5 min), then 30 high stringency cycles; 94°C (1 min), 60°C (1 min), and 72°C (2 min). The primers tested were the reverse sequencing primer (RSP), GGAAACAGTCATGACCATGA; LC, CCATGCGCATGCATGAGA; LD, CCACACGCGCACACGGGA; LE, CCGCACGCGCACGCAAGG; LF, CCACGCGTGTGTGTGAAA; and ZF9, AGAGAGAAACCCACCAGA. Ten microliters of 80% formamide, with bromophenol blue and xylene cyanol dyes, was added to 2.5 μl of each PCR sample. The samples were heated to 65°C for 15 min and 2.5 μl of each was loaded on a standard 5% polyacrylamide/50% urea sequencing gel prepared in 1× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) and electrophoresed at 1500 V until the xylene cyanol dye had reached the bottom of the gel. The gel was dried under vacuum onto 3MM paper (Whatman). The RAP fingerprint was visualized by autoradiography using Kodak XAR film.

Isolation and Cloning of RAP Products. The autoradiogram was aligned with the gel using radioactive ink dots and bands were cut from the gel using a razor blade. The piece of acrylamide was placed in a centrifuge tube and the DNA was eluted for at least 1 hr at 65°C into 50 μl of TE. Five microliters of the eluent was PCR amplified in 50 μl using the standard PCR protocol (Perkin–Elmer/Cetus), 0.25 μCi of [α-^{32}P]dCTP, and the same primer. The amplified material was checked against the initial RAP reaction by running a second polyacrylamide gel. The products were blunt end cloned by standard methods (20) into the *Srf* I site of the pCR-script SK(+) vector (Stratagene), mobilized into single-stranded phage, and sequenced using the Sequenase kit (United States Biochemical).

PFGE. Genomic DNA from a late logarithmic phase *S. typhimurium* LT2 culture was prepared in InCert agarose (FMC) as described (17, 18). The DNA was cleaved with 10 units of *Bln* I (Takara Biochemical, Berkeley, CA) or *Xba* I (Stratagene) for 4 hr in 1× KGB (22), with potassium acetate replacing potassium glutamate. The fragments were separated using a TAFE system (Beckman) and 0.5× TAE buffer (40 mM Tris-acetate/1 mM EDTA). After electrophoresis, the gel was treated with 0.2 M HCl for 15 min, with denaturation solution (0.5 M NaOH/1.5 M NaCl) for 30 min, and finally with neutralization buffer (0.5 M Tris, pH 7.5/2.5 M NaCl) for 30 min. DNA was transferred to a Duralon-UV membrane (Stratagene) using 20× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate) buffer and then UV cross-linked.

Dot Blot Analysis of Induced Mu*d*-P22. Strains carrying Mu*d*-P22 at different chromosome map locations were induced by mitomycin C as described (14). Preparation of DNA for the mapping array was largely as described (17). DNAs were prepared from each induced phage, dot blotted to an 11 × 10 cm Duralon-UV membrane, and UV cross-linked.

Probe Preparation and Hybridization. The 23S rRNA-specific oligonucleotide 5′-GGTGTCGACTATGAACCTGCTTCCCATCGACTAC-3′ was radiolabeled using [γ-^{32}P]ATP and polynucleotide kinase (Stratagene) and used as a control in Northern blots for quantitation of RNA. Hybridizations to RAP blots, PFGE blots, and Northern blots were performed with 2–5 × 10^8 cpm/μg of [α-^{32}P]dCTP-labeled probes that were made using a random priming kit (Stratagene). For Southern blotting of RAP fingerprints, DNA was transferred directly from the polyacrylamide gel after rehydration in 20× SSC. The DNA was then Southern transferred to a membrane (Duralon-UV, Stratagene) by capillary action using 20× SSC buffer and UV cross-linked. Blots were prehybridized with 5× SSC, 0.5% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, and 0.02% SDS at 65°C for 4 hr. Hybridization took place in the same buffer at 70°C overnight (65°C for Northern blots). After hybridization, the membranes were washed with 2× SSC/0.1% SDS twice at room temperature for 15 min and washed with 0.1× SSC/0.1% SDS at 65°C for 15 min. For the 23S rRNA-specific oligonucleotide, the hybridization and washing temperatures were 37°C.

RESULTS AND DISCUSSION

RNA Fingerprinting. First strand cDNA is synthesized with Moloney reverse transcriptase using an arbitrarily selected primer. Primer extension occurs most favorably at better matches and becomes progressively less likely at worse matches. The arbitrarily primed first strand cDNA is heat denatured and subjected to arbitrarily primed second strand synthesis using Taq polymerase at low stringency. Those products that have the primer sequence at both ends are then PCR amplified at high stringency with simultaneous radiolabeling. Products are separated by polyacrylamide gel electrophoresis and visualized by autoradiography. The resulting fingerprint patterns are highly reproducible. Each fingerprint yields a pattern of 10 or more clearly visible PCR products. At least two concentrations must be used for each RNA sample to control for slight concentration effects. The method requires only a few nanograms of total cellular RNA for each primer.

The RAP products that are differentially amplified between RNA samples generally reflect differential gene expression. The relative amounts of an amplified product measured by RAP closely parallel the relative amounts of RNA in the various samples as measured by Northern blots (1, 5). Source-specific differences in mRNA abundance revealed by

RAP are useful for studying differential gene expression and the method is applicable to the detection of transcriptionally regulated RNAs in a wide variety of situations. The primers select mRNAs for fingerprinting based partly on how well the primer matches with template. Thus, the 10 or more RAP products in each lane are obtained as if they were each picked at random from a hypothetical partly normalized cDNA library and then each used as a probe on Northern blots.

Some of the RAP products may be derived from the abundant structural RNAs. However, because these RNAs are generally not differentially expressed, their presence as unvarying products in the fingerprint does not interfere with the experiment. Occasionally, the largest RAP products in the fingerprint are unreliably amplified, so these should be ignored even if they seem to show variation.

Six fingerprints of RNAs from logarithmically growing bacterial cultures, treated and untreated with H_2O_2, were generated by the RAP strategy using six different 18-base primers (*Materials and Methods*). For each primer there were 10 or more observable bands in each lane but only a few differentially amplified cDNA products were observed. Fig. 1 shows an example of fingerprints using RSP and ZF9 primers. Examples of differentially amplified products are indicated by arrowheads.

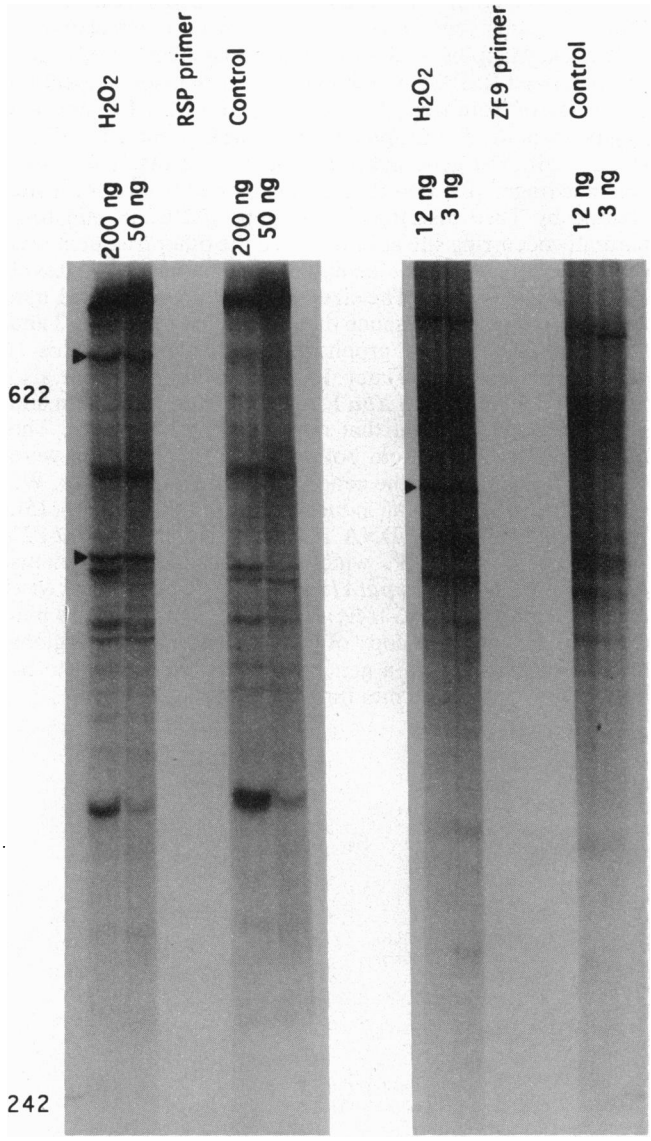

FIG. 1. RAP fingerprinting of RNAs. Total RNA from cells in logarithmic phase and after H_2O_2 stress were RAP fingerprinted with primers RSP and ZF9 at different RNA concentrations, as indicated. The products were resolved on a 5% polyacrylamide/50% urea/1× TBE gel and visualized by autoradiography. A portion of the gel is shown. Arrowheads indicate differentially amplified products. Lengths are shown in nucleotides on the left.

The differentially amplified RAP products were isolated and cloned in the manner previously described (1). The clone RSP435 contained a 435-bp cDNA insert (GenBank accession no. U04161) derived from RNA isolated from peroxide-treated cells using the RSP primer. This product was not visible in the RAP products derived from RNA isolated from untreated cells. The longest potential open reading frame extended for 128 amino acids from one end (excluding the primer sequence) and terminated 51 bases from the other end of the clone. A search of the GenBank data base revealed no significant homology with any previously sequenced DNA or protein. One other peroxide-inducible cDNA has been cloned and sequenced (data not shown). This clone has homology to the "U" tail gene of bacteriophage λ and is likely to be derived from the lambdoid prophage *Fels*-1 (17, 23, 24). This prophage may be induced in some cells by the level of peroxide used in these experiments. The gene has not been further characterized.

To confirm that RSP435 corresponded to the differentially amplified RAP product and that differential amplification was reproducible, a separate fingerprinting experiment using the same primer was performed. This fingerprint was transferred onto a Duralon-UV membrane and probed with the clone. RSP435 hybridized to a product of 435 bases in the lanes derived from the *S. typhimurium* LT2 cultures treated with H_2O_2 for 30 min and 120 min and did not hybridize in lanes of the fingerprint derived from untreated cultures or from the

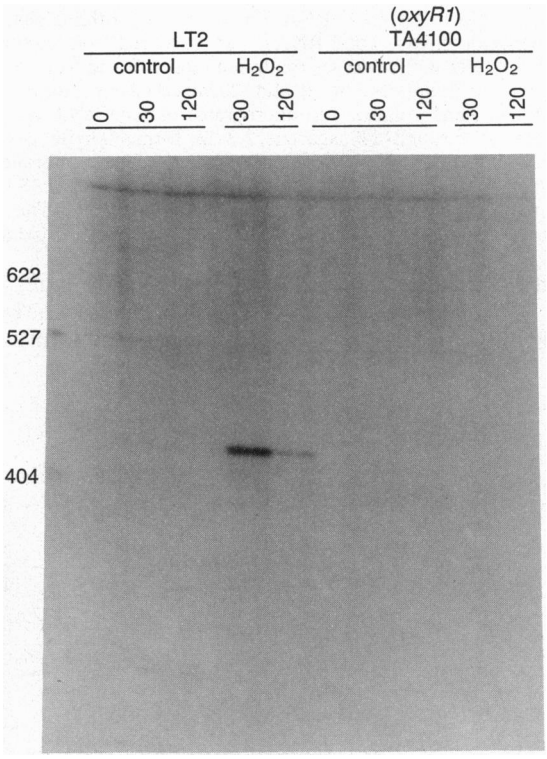

FIG. 2. Southern blot of RAP fingerprint. The RSP primer was used to fingerprint 200 ng and 50 ng of total RNA from LT2 and *oxyR1* cells. RNA was isolated from cultures in mid-logarithmic phase and from cells during peroxide treatment at 0, 30, and 120 min. The RAP products were separated by electrophoresis on a 5% polyacrylamide/50% urea/1× TBE gel and then transferred to a membrane and hybridized with the radiolabeled RSP435 clone. Lengths are shown in nucleotides on the left.

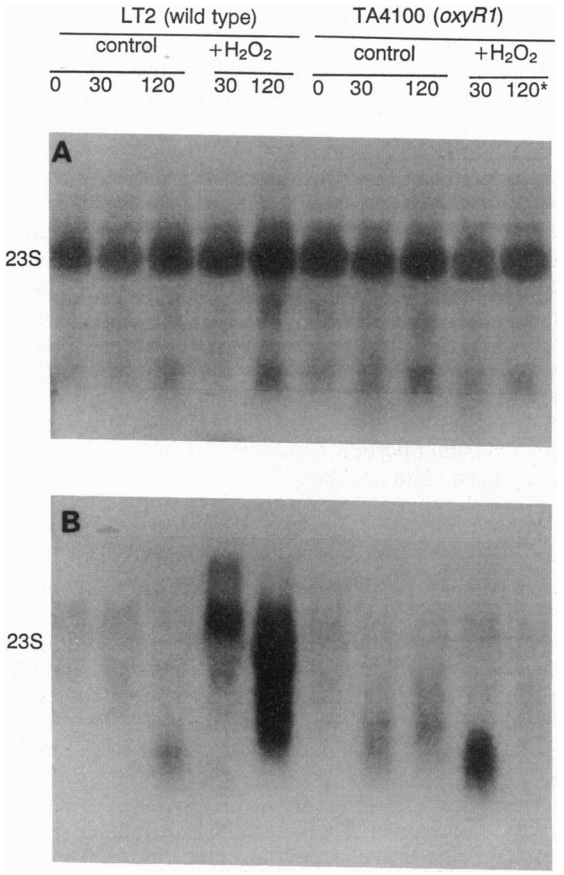

FIG. 3. Northern blot of RNA from cells in logarithmic phase and after peroxide stress. Total RNAs were prepared from cultures of LT2 and *oxyR1* without H_2O_2 treatment (control) and from cultures with peroxide treatment at 0, 30, and 120 min. At 0 min, cultures were at mid-logarithmic phase. Ten micrograms of each RNA was electrophoresed through 1% agarose/2.2 M formaldehyde gels and transferred to a Duralon-UV membrane. (*A*) The membrane was hybridized with an oligonucleotide complementary to the 23S rRNA used to control for the amount of RNA loaded. (*B*) The same membrane was hybridized with the radiolabeled RSP435 clone.

strain TA4100 (*oxyR1*) that is resistant to H_2O_2 stress (12) (Fig. 2). Note that the level of RSP435 began to decline by 120 min in this experiment as the bacteria recovered from stress.

Stress Induction of the RSP435 RNA Shown by Northern Blotting. Northern blots were performed to confirm that this differentially amplified RAP product RSP435 represented a differentially expressed RNA (Fig. 3). RNA hybridizing to the RSP435 clone was induced manyfold after 30 min in LT2 but was not induced in an *oxyR1* mutant. This result is consistent with the result obtained by the Southern blot of the RAP fingerprint in Fig. 2.

The growth curve for the *oxyR1* mutant was unaffected by peroxide treatment, whereas the rate of growth of the LT2 wild-type cells was transiently diminished (data not shown). The dominant *oxyR1* mutant constitutively overexpresses at least 9 H_2O_2-inducible genes. However, at least 21 other peroxide-inducible genes are not expressed (12). These latter genes are presumably induced by a separate, perhaps more general, stress response. RSP435 could be in this class. RSP435 is probably not a part of the other known stimulon induced in the macrophage phagosome *phoP/phoO*, because acidification rather than peroxide is implicated as the intracellular inducer of this regulon (25). Whether RSP435 responds to other regulators has not yet been investigated.

Physical Mapping of the Corresponding Gene. We further characterized RSP435 by mapping its chromosomal location by means of Southern blots to *Bln* I and *Xba* I restriction fragments of the *S. typhimurium* genome separated by PFGE (17, 18, 26). The gene hybridized at high stringency to two genomic fragments. One fragment extended from a *Bln* I site created by Tn*10* insertion in the gene *gal* at 18 min to a naturally occurring site at 21 min, and the other fragment was generated by cleavage at the naturally occurring *Bln* I sites at 38 and 57 min (Fig. 4). The size of the fragment at 18–21 min is dependent on the presence (lane 3) or absence (lanes 2 and 3) of the 50-kb *Fels*-1 prophage in the fragment. This is discussed in more detail later. In Fig. 4, lane 1, the clone also hybridized to an 800-kb *Xba* I fragment from 13–26 min and a 35-kb *Xba* I fragment that maps to 56–57 min (26). The hybridization signals from both places in the genome were similar, indicating that the genes are highly homologous. We also probed DNA from an induced array of Mu*d*-P22 (14, 15). RSP435 hybridized to DNA induced from three Mu*d*-P22 insertions: *aroA*::Mu*d*P, which packages 42-kb segments clockwise from 19 min; *putA1019*::Mu*d*P, counterclockwise from 21.5 min; and *purG2149*::Mu*d*P, clockwise from 54 min (data not shown). One copy of the gene and flanking regions has been isolated from a genomic library and shown to be adjacent to *pncB* at 20 min (unpublished data).

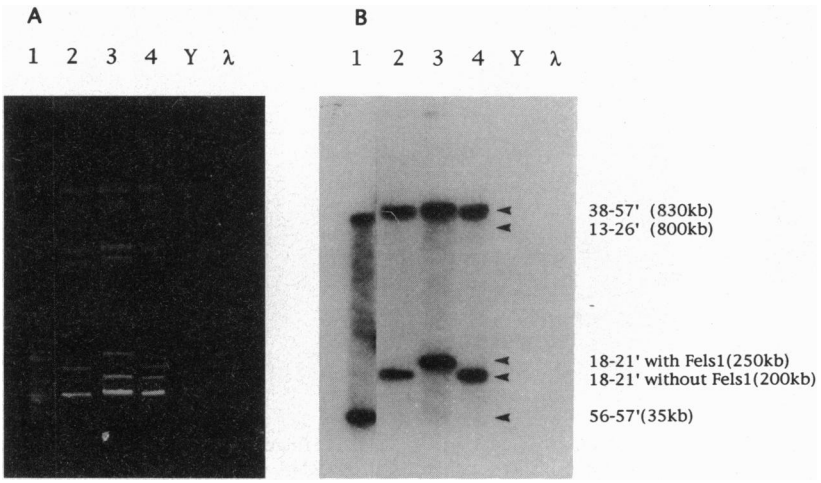

FIG. 4. Physical mapping of RSP435 on the *S. typhimurium* genome. Genomic DNA was cleaved with *Bln* I or *Xba* I and separated using a TAFE system. (*A*) Ethidium-stained gel. (*B*) Southern blot of the same gel probed with radiolabeled RSP435. Lanes: 1, LT2 wild type cleaved with *Xba* I; 2, KKWQ100F2 cleaved with *Bln* I; 3, KKWQ100F1 cleaved with *Bln* I; 4, KKWQ100 cleaved with *Bln* I; Y, yeast chromosome markers; λ, λ phage DNA in 48.2-kb concatemers. Lengths are given in kb on the right.

The prophages *Fels*-1 and *Fels*-2 in *S. typhimurium* also map near to both of the regions to which RSP435 hybridizes (ref. 17; K.K.W., N. Yamamoto, and M.M.). Genomic DNAs of *Fels*-1, *Fels*-2 lysogenic *S. typhimurium*, and strains cured of *Fels*-1 and *Fels*-2 (23, 24) were cleaved with *Bln* I and *Xba* I and the fragments were separated by PFGE and then Southern blotted. RSP435 still hybridized to strains cured of the prophage, eliminating these prophage as sources of the RSP435 gene (Fig. 4, lanes 2–4).

The regions to which RSP435 homologs mapped do not contain any previously known inducible genes. Two tRNA-serine loci that map to similar locations as RSP435 in *Escherichia coli* (27) have no homology with RSP435. So far, the best candidate loci for RSP435 are the "leaky" complementation groups *lykA* and *lykB* that map to ≈20 min and *lykC* and *lykD* that map to ≈58 min (28).

In summary, an entirely physical strategy for characterizing conditionally expressed genes was employed to identify a stress-inducible gene in a bacterium: RAP, cloning, sequencing, Northern blots, and PFGE. Because RAP can identify differentially expressed genes without the need to alter the genome, the disruption of natural physiological responses is not a concern. RAP should be of particular utility for characterizing conditionally expressed genes in organisms in which genetic methods are difficult or have not been developed.

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